Regulation of chemokines and IL-6 production in human mesangial cells: Potential role in glomerulonephritis

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REGULATION OF CHEMOKINES AND IL-6 PRODUCTION IN HUMAN MESANGIAL CELLS : POTENTIAL ROLE IN GLOMERULONEPHRITIS

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CONTENTS

ABSTRACT

'Activated' mesangial cells (MC) may play a direct role in mediating glomerular inflammation via the generation of inflammatory mediators. The potential of human MC to express chemokines, and the molecular mechanisms regulating MC cytokine production were investigated. In addition to the neutrophil chemoattractant IL-8 and monocyte chemoattractant MCP-1, cultured MC produce the chemotactic factor for CD45RO/CD4$^+$ T lymphocytes, RANTES following stimulation with IL-1 or TNF. The kinetics and specificity of RANTES expression was markedly different to that of IL-8 or MCP-1, RANTES was expressed later and TNF was the more potent stimulant. A difference in the kinetics of chemokine expression following IL-1 or TNF stimulation was also evident.

The intracellular second messenger, cAMP differentially regulated MC cytokine production. cAMP-elevating agents upregulated IL-1 induced IL-6, downregulated TNF induced RANTES and had no effect on IL-1 or TNF induced IL-8 production. The regulatory effects of cAMP on IL-1 induced IL-6 were occurring at the level of gene expression. cAMP did not modify IL-6 mRNA stability indicating the mechanism of regulation was at the level of transcription.

Pretreatment of MC with pertussis toxin (PT), an agent widely employed as a tool to investigate the role of heterotrimeric $G_{i/o}$ type proteins in signalling mechanisms, resulted in partial inhibition of IL-1 induced IL-8 and IL-6 production. The purified B oligomer of PT and mutated forms of the toxin however, were equally inhibitory indicating the mechanism of PT inhibition was independent of G protein modification by ADP-ribosylation.

Inducible nitric oxide synthase (iNOS) mRNA and protein expression was observed in human MC after stimulation with a combination of cytokines, IL-1 and IFN-γ, single cytokines were ineffective. Production of NO however, could not be readily detected, indicating complex regulation of iNOS activity. Furthermore, the specificity of iNOS induction in human MC indicates NO may not play a role in IL-1 signalling for chemokine expression.

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My final thanks go to my parents for their unending support. Who would have thought undertaking a PhD would lead to Dad liking horses!

LIST OF FIGURES AND TABLES

ABBREVIATIONS

AcD	Actinomycin D
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BH_4	tetrahydrobiopterin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-5'- monophosphate
C-C CKR	C-C chemokine receptor
cGMP	Cyclic guanosine-5'- monophosphate
CHO	Chinese hamster ovary cells
CHX	Cycloheximide
Con-A	Conconavalin A
CINC	Cytokine-induced neutrophil chemoattractant (rat homologue of IL-8)
CSF-1	Colony-stimulating factor-1
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5-chloro)tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
CT	Cholera toxin
CT-B	Cholera toxin B oligomer
CTAP-III	Connective tissue activating protein III
CTP	Cytosine triphosphate
DAG	Diacylglycerol
DAN	2,3-Diaminonaphthalene
DARC	Duffy antigen receptor for chemokines
Db-cAMP	Dibutyryl cyclic adenosine monophosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagles medium
DMF	Dimethylformamide

DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ENA-78	epithelial-derived neutrophil attractant-78
FAD	Flavin dinucleotide
FCS	Foetal calf serum
FITC	Fluorescein isothiocynate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FMN	Flavin mononucleotide
GBM	Glomerular basement membrane
GCP-2	Granulocyte chemotactic protein-2
GDP	Guanosine diphosphate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GN	Glomerulonephritis
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HMAP	4-hydroxy-3-methoxyaceto-phenone
[^{125}I]	Iodinated radiolabel
IBMX	3-isobutyl-1-methyl-xanthine
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor-1
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ-inducible protein 10
LFA-1	Leukocyte function antigen-1
L-NAA	N^{ω}-amino-L-arginine

L-NMMA	N^{ω}-monomethyl-L-arginine
L-NNA	N^{ω}-nitro-L-arginine
LPS	Lipopolysaccharide
LT	Leukotriene
MAP kinase	Mitogen-activated protein kinase
MC	Mesangial cell
MCP-1	Monocyte chemotactic protein-1
MGSA/*gro*	Melanocyte growth stimulatory activity
mig	Monokine induced by IFN-γ
MIP-1α	Macrophage inflammatory protein-1α
MOPS	3-[N-morpholino]propane-sulphonic acid
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP-2	Neutrophil activating peptide-2
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
NTN	Nephrotoxic serum nephritis
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
[^{32}P]	Phosphate radiolabel
PAF	Platelet activating factor
PBP	Platelet basic protein
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidinedithiocarbamate
PF4	Platelet factor 4
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PKA	Protein kinase A

PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl fluoride
PT	Pertussis toxin
PT-B	Pertussis toxin B oligomer
RANTES	Regulated on activation, normal T cell expressed and secreted
RIA	Radioimmunoassay
SDF-1α	Stromal cell-derived factors-1α
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOD	Superoxide dismutase
TEMED	N, N, N', N'-tetramethylethylene diamine
β-TG	β-thromboglobulin
TGFβ	Transforming growth factor-β
TNF	Tumour necrosis factor
TNF-R1	Tumour necrosis factor receptor 1
TNF-R2	Tumour necrosis factor receptor 2
TRIS	Tris-(hydroxymethyl)-methylamine
Tween-20	Polyoxyethylenesorbitan monolaurate
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
VLA_4	Very late antigen-4

CELL LINES:

EL4 cells	Murine thymoma cell line
FS-4 fibroblasts	Human foreskin fibroblast cell line
G361 cells	Human melanoma cell line
HL-60 cells	Human myelocytic cell line
HT-29 cells	Human colonic epithelial cell line
PC60 cells	Hybrid of a murine T cell clone with a rat thymoma line
Swiss 3T3 fibroblasts	Murine fibroblast cell line

U937	Human pre-monocytic cell line
YT lymphocytes	Human natural killer-like T cell line
70Z/3 cells	Murine pre-B cell line

1. INTRODUCTION

1.1. GLOMERULONEPHRITIS

Glomerulonephritis (GN), a major cause of renal failure, is a general term applied to a spectrum of disorders that may essentially be considered as immunologically mediated injury to glomeruli (Couser, 1993). Although the number of patients receiving dialysis and transplantation continues to increase by approximately 10% each year, understanding of the pathogenesis and treatment of GN remains at best rudimentary despite decades of research in this field. Consequently most of the therapies that are used are non-specific with major side effects and offer limited therapeutic benefits for the patient (Schlöndorff *et al.* 1995).

An initial step in the induction of glomerular injury is the formation of immune complexes within the glomeruli. These may be formed by a number of different mechanisms, including deposition of circulating immune complexes, trapping of circulating free antigen and subsequent immune complex formation *in situ* or production of antibody to normal antigens in the glomerular basement membrane (GBM) or on glomerular cell membranes (Couser, 1993). In the majority of patients with immune complex mediated GN, the nature of the antigen involved is not known. Exogenous antigens may be derived from viruses, bacteria (e.g. post-streptococcal GN), parasites or drugs, while endogenous antigens may include host DNA (e.g. systemic lupus erythematosus), an antigen in the glomerular basement membrane (e.g. anti-GBM nephritis) or the GP330 protein present on the membrane of glomerular epithelial cells (e.g. experimental membranous nephropathy). Increasing evidence suggests that many more of the antibody-mediated human glomerular diseases are autoimmune in nature than previously suspected, thus genetic factors may play a major role in determining the incidence and severity of GN (Burns *et al.* 1991).

Regardless of how the immune deposits form in glomeruli, they can cause injury by two distinct types of mechanisms, depending in part on where the deposits form. One is an

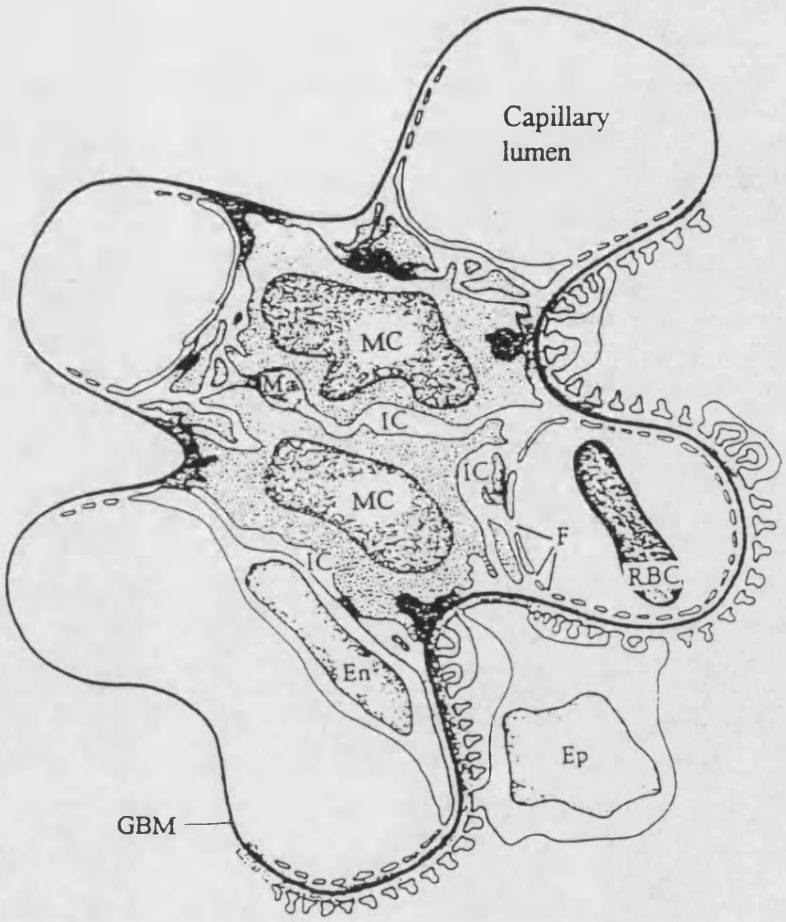


Figure 1. Schematic representation of the glomerulus. This cross section shows the central core of the glomerulus containing mesangial cells (MC) embedded in extracellular matrix (Ma), which contains intercellular channels (IC). A single endothelial cell (En) lines each capillary and fenestration (F) of endothelium (En) allows continuity between capillary lumen and intercellular channels. Epithelial cells (Ep) are located outside the glomerular basement membrane (GBM), which incompletely surrounds the endothelial wall of the capillary loops. (RBC = red blood cell) (From Mene *et al*, 1989).

A key role for leukocytes in mediating glomerular injury is based on evidence from animal models of GN demonstrating that leukocyte depletion prior to the induction of GN attenuates renal injury (Schreiner *et al.* 1978; Tucker *et al.* 1985; Lavelle *et al.* 1981). Furthermore, immunohistochemial analysis employing monoclonal antibodies specific for leukocyte antigens have shown the presence of neutrophils, macrophages and T lymphocytes in many human forms of GN (Rovin & Schreiner, 1991), as well as in experimental models (Radounikli *et al.* 1995; Main *et al.* 1992). Typically, macrophages were found in much greater numbers than T lymphocytes in inflamed glomeruli (Rovin & Schreiner, 1991; Main *et al.* 1992). An investigation of the kinetics of leukocyte infiltration in rats with accelerated nephrotoxic serum nephritis (NTN), an experimental model of anti-glomerular basement membrane nephritis (Schreiner *et al.* 1978; Unanue & Dixon, 1965) demonstrated a transient infiltration of neutrophils and a sustained increase in monocytes using immunohistology and flow cytometry (Tam *et al.* 1996). Neutrophil influx was maximal 6 hours after disease induction and returned to baseline levels by 24 hours, while monocyte influx was maximal at 24 hours and persisted thereafter (>96 hours). Similarly, in the anti-Thy-1 model of mesangioproliferative GN in rats (Yamamoto & Wilson, 1987), monocyte/macrophage infiltration was pronounced within 30 minutes of antibody injection and was maintained up to 3 weeks after the induction of GN (Stahl *et al.* 1993). Infiltrating T lymphocytes have been observed in glomeruli at 24 hours (Tipping *et al.* 1985) or 10 days (Radounikli *et al.* 1995) after induction of immune complex mediated GN in rats.

Once localised in tissue, leukocytes mediate glomerular injury via the local release of inflammatory mediators. Neutrophils release oxidants and proteases which directly damage the surrounding tissue (Johnson *et al.* 1994), while macrophages are a rich source of a variety of inflammatory mediators including oxidants, prostaglandins, leukotrienes, cytokines, polypeptide growth factors, complement components and coagulation factors (Nathan, 1987). The role of T lymphocytes is less clear. However, the T cell influx appears to be predominantly of the T helper cell subset ($CD4^+$) which suggests an involvement in both the initiation and progression of the disease via the release of inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-

4 and granulocyte/macrophage-colony stimulating factor (GM-CSF) that amplify the immune response to a specific antigen (Main *et al.* 1992).

1.1.2. Role of cytokines in glomerulonephritis

Cytokines are locally active, inducible polypeptides of molecular weight greater than 5 kDa which cause specific, receptor mediated effects in target cells and/or in the cytokine producing cells themselves. The cell to cell communication via released cytokines is a key event in the initiation and development of inflammatory diseases (Sedor, 1992). Tissue injury elicits a network of cytokines and growth factors and as most cytokines are multifunctional, both redundancy and specificity exists within the network (Akira *et al.* 1990a). The resulting tissue response ultimately reflects an integration of the molecular mechanisms initiated by cytokine interactions.

The expression of cytokines and/or their specific receptors in glomeruli of animals or patients undergoing renal injury has suggested their involvement in the pathogenesis of experimental and human glomerular disease (Sedor, 1992). Cytokines are involved in both the early and late stages of GN, with proinflammatory cytokines such as IL-1 and tumour necrosis factor (TNF) important in initiating the disease, while growth factors such as platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ) may play a role in its progression to sclerosis, characterized by mesangial cell proliferation and extracellular matrix expansion (Isaka *et al.* 1993).

1.1.2.1. *Interleukin-1 and tumour necrosis factor*

IL-1 and TNF are pleiotropic cytokines which are involved in the regulation of the immune response, hematopoiesis, and inflammation (Le & Vilcek, 1987). IL-1 is one of the most potent and multifunctional activators of cells so far described (O'Neill, 1995), and despite being biochemically and immunologically distinct proteins, IL-1 and TNF show striking similarity in the broad range of biological activities they induce (Le & Vilcek, 1987). Both cause acute inflammation when injected locally *in vivo*, and have similar proinflammatory activities *in vitro*. Several of the activities of IL-1 and TNF are due to the induction of secondary proteins in the target cells.

IL-1 and TNF are synthesized by monocytes and macrophages, and while these cells are thought to be the principle cellular source of TNF, IL-1 production has been demonstrated in a wide range of immune and non-immune cells, including epithelial, endothelial and mesangial cells (Oppenheim *et al.* 1986). Three forms of IL-1 have been identified, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra). IL-1α and IL-1β have 26% homology at the amino acid level. Both forms bind to the same cell surface receptors and induce similar biological activities (Le & Vilcek, 1987). The principal difference between the two forms is that IL-1α is membrane bound, while IL-1β, the predominant form, is released into the surrounding medium (Abbott *et al.* 1992). IL-1ra is structurally related to IL-1α and IL-1β, but binds to the IL-1 receptors without inducing any discernible biological response and thus functions as a naturally occurring, specific receptor antagonist (Arend, 1991). TNF is structurally and functionally related to lymphotoxin, a protein produced by activated T lymphocytes, which binds to and signals via the TNF receptors (Fiers, 1991). Lymphotoxin is sometimes referred to as TNFβ, while the 'original' TNF is termed TNFα.

A number of studies have implicated an involvement of IL-1 and TNF in the pathogenesis of experimental and human glomerular injury (Sedor, 1992; Baud & Ardaillou, 1994). Steady state IL-1 and TNF mRNA expression was increased in renal cortex from mice with experimental nephritis (Boswell *et al.* 1988). Immunohistochemical analysis demonstrated IL-1 and TNF positive cells within the mesangium of glomeruli during acute aminonucleoside nephrosis, which appeared to originate mainly from infiltrating macrophages (Diamond & Pesek, 1991). Furthermore, glomeruli purified from rat kidneys with experimentally induced nephritis have been found to release IL-1 and TNF when incubated *ex vivo* (Baud & Ardaillou, 1994; Matsumoto & Hatano, 1995), and cytokine production was dependent on macrophage infiltration (Tipping *et al.* 1991). Finally, administration of anti-TNF antibody (Mulligan *et al.* 1993; Hruby *et al.* 1991) or IL-1ra (Lan *et al.* 1993) resulted in reduced glomerular injury in experimental anti-GBM nephritis.

1.1.2.2. *Interleukin-6*

IL-6 is another multifunctional cytokine which has been found to have growth and differentiation activities on a wide variety of tissues and cells. The biological activities of IL-6 include stimulation of B-cell differentiation, induction of acute phase proteins in liver cells, growth-promotion of myeloma cells and the induction of IL-2 and IL-2 receptor expression, proliferation and differentiation in T cells. Many cells are able to synthesize IL-6 after appropriate stimulation, and major cellular sources are monocytes/macrophages, fibroblast and endothelial cells (Hirano *et al.* 1990).

IL-6 has been implicated in the pathogenesis of mesangioproliferative GN (Horii *et al.* 1993). This type of GN is histologically characterized by proliferation of resident glomerular MC, suggesting the involvement of a MC growth factor in the pathogenesis of the disease. IL-6 was shown to induce proliferation of cultured rat MC (Horii *et al.* 1989; Ruef *et al.* 1990), and may function as an autocrine growth factor, since rat and human MC *in vitro* produce IL-6 upon appropriate stimulation (Horii *et al.* 1989; Ruef *et al.* 1990; Abbott *et al.* 1991; Brown *et al.* 1991a). *In vivo* evidence comes from patients with mesangioproliferative glomerulonephritis showing in 50% of the cases significantly elevated urinary IL-6, which was not detected in healthy volunteers or patients with other glomerulopathies (Horii *et al.* 1989). A positive correlation between urinary IL-6 and the severity of the disease was observed in the study. Immunostaining of biopsy samples from patients with proliferative GN demonstrated IL-6 production, which was mainly localised in the glomerular mesangium (Horii *et al.* 1989). Furthermore, overexpression of IL-6 in transgenic mice (C57BL/6), produced by fusing the human IL-6 genomic gene with the immunoglobulin heavy chain enhancer (Eμ), resulted in development of mesangioproliferative GN, along with massive plasmacytosis (Suematsu *et al.* 1989).

In addition to IL-6, a number of other cytokines and growth factors have been implicated as MC mitogens, or co-mitogens. These include IL-1, insulin-like growth factor 1 (IGF1), basic fibroblast growth factor (bFGF), PDGF and epidermal growth factor (EGF) (Kashgarian & Sterzel, 1992). PDGF has been reported to induce the proliferation of cultured rat (Shultz *et al.* 1988) and human (Floege *et al.* 1991) MC, and

platelet depletion prior to the induction of experimental mesangioproliferative GN was found to partially reduce proliferation of resident glomerular cells (Johnson *et al.* 1990).

In contrast to the potential pathological role of IL-6 in mesangioproliferative GN, a recent study has suggested IL-6 may have therapeutic effects in other forms of glomerular injury. Pretreatment of rats with IL-6 did not exacerbate glomerular injury in the NTN model of GN, instead it largely abrogated the ability of lipopolysaccharide (LPS) to enhance injury (Karkar *et al.* 1993). An ability of IL-6 to down-regulate IL-1 and TNF synthesis by LPS stimulated macrophages *in vitro* (Aderka *et al.* 1989; Schindler *et al.* 1990) may explain the anti-inflammatory properties of IL-6 in this model, and IL-6 treatment was associated with a marked reduction in glomerular IL-1 and TNF mRNA expression.

1.2. CHEMOKINES

The migration of leukocytes from the circulation to an extravascular site of injury is a multi-step process that requires a series of co-ordinated signals, including the expression and activation of cellular adhesion molecules and the generation of chemotactic gradients by the cells of the extravascular compartment (Adams & Shaw, 1994). Recent advances in understanding leukocyte migration have come from the discovery of a superfamily of chemotactic factors, collectively known as the chemokines (Lindley *et al.* 1993; Baggiolini *et al.* 1994). Members of this family are small (<10 kDa), basic, heparin-binding proteins which are structurally related by possessing four conserved cysteine residues that form two disulphide bonds and establish the tertiary structure of the protein. The chemokines are divided into two sub-families based on the position of the first two cysteine residues in the molecule (Fig. 2). In the C-X-C sub-family, the first two cysteine residues are separated by an intervening amino-acid and the genes encoding this group of proteins are clustered on human chromosome 4. The C-C sub-family possess adjacent cysteine residues and are clustered on human chromosome 17. The key feature of this family is their ability to attract subsets of leukocytes in a relatively specific manner. In general the C-X-C chemokines, of which IL-8 is a

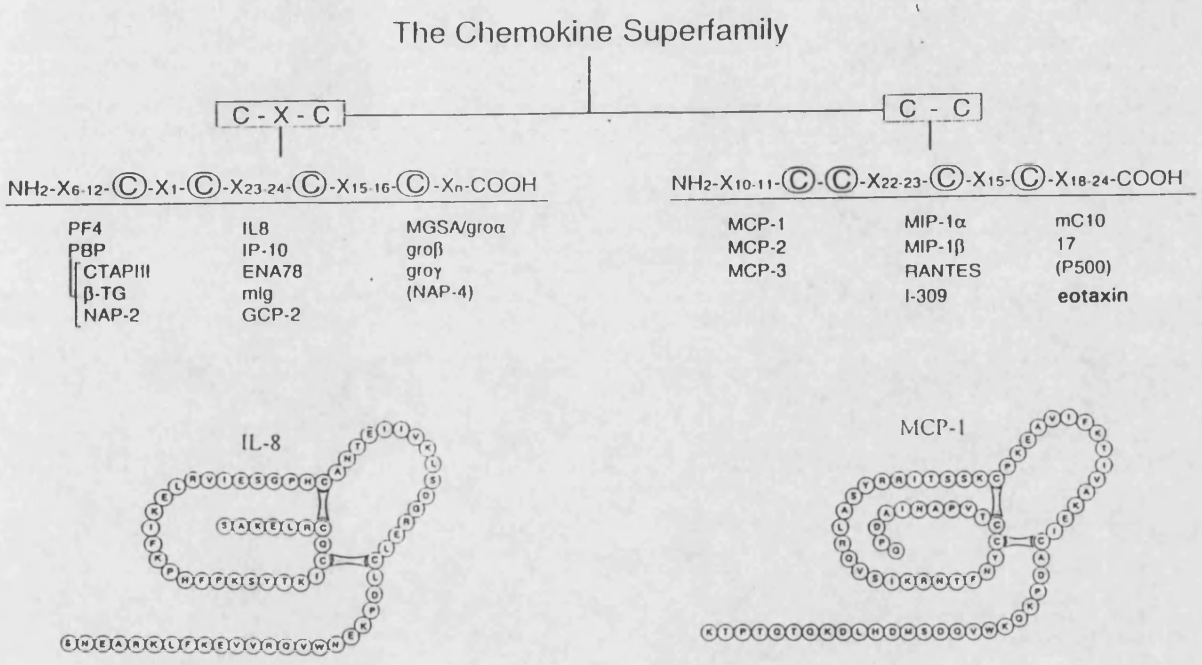


Figure 2. Organisation of the chemokine superfamily. Diagrammatic representation of the mature protein structures of C-X-C and C-C chemokines. The number of intervening amino acids between the cysteine residues in a typical human chemokine is denoted by the subscript. The designations for the molecules listed are those of the human chemokines, except for murine C10 and 17 where no human molecule has yet been identified. The line connecting CTAP III, β-TG and NAP-2 to PBP indicates that these proteins are thought to be derived from a common precursor. (NAP-4) and (P500) are chemokine variants that lack some of the cysteines in the characteristic four-cysteine motif. In the diagram showing the two loop structure of human chemokines, the amino acids in one letter codes are A=Ala; C=Cys; D=Asp; E=Glu; F=Phe; G=Gly; H=His; I=Ile; K=Lys; M=Met; N=Asn; P=Pro; Q=Gln; R=Arg; S=Ser; T=Thr; V=Val; W=Trp; Y=Tyr (Modified from Schall *et al*, 1994, Baggiolini *et al*, 1994).

prominent member (Baggiolini *et al.* 1989), attract and activate neutrophils, while the C-C molecules, which include monocyte chemotactic protein-1 (MCP-1) and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) preferentially act on mononuclear cells (Table 1). MCP-1 is a potent chemoattractant for monocytes/macrophages (Leonard & Yoshimura, 1990), and more recently was been found to be chemotactic for $CD4^+$ and $CD8^+$ T lymphocytes (Loetscher *et al.* 1994; Taub *et al.* 1995). In addition to monocytes, RANTES is a selective chemotactic agent for $CD45RO/CD4^+$ T cells (memory T cells) (Schall *et al.* 1990) and eosinophils (Kameyoshi *et al.* 1992) (Table 2). Unlike the C-C sub-family, the C-X-C chemokines can be further divided into proteins which contain or lack an ELR motif. The ELR motif represents three amino acids, Glu-Leu-Arg that immediately precede the first cysteine residue in the molecule. This motif is important in receptor/ligand interactions on neutrophils, thus chemokines which lack this motif (PF4, IP10 and Mig) are only weak neutrophil chemoattractants. Production of the chemokines is not limited to immune cells, with a spectrum of tissue cells (i.e. fibroblasts, endothelial cells and MC) capable of expressing these cytokines upon appropriate stimulation (e.g. IL-1, TNF, LPS) (Baggiolini *et al.* 1994).

A new class of chemokine, designated the C sub-family has recently been proposed, following the discovery of lymphotactin (Kelner *et al.* 1994). Lymphotactin is structurally similar to members of both the C-C and C-X-C sub-families, but lacks two of the four characteristic cysteine residues (lacks cysteines 1 and 3). Furthermore, the gene encoding lymphotactin is located on human chromosome 1. Lymphotactin is unique in that it is chemotactic for lymphocytes, but not monocytes or neutrophils, and lymphotactin is abundantly produced by $CD8^+$ T cells.

Chemokines participate at several levels of the multi-step process that regulates leukocyte migration into injured tissue. The migration process is defined by four sequential steps: rolling, triggering, adhesion and migration (Fig. 3) (Adams & Shaw, 1994). Rolling interactions between leukocytes and endothelium cause circulating cells to slow their flow and roll along the vessel wall. Rolling is mediated by selectins, which are expressed on both leukocytes and endothelium. This initial adhesive

Neutrophils		Monocytes	
PF4	(C-X-C)	MCP-1	(C-C)
IL-8	(C-X-C)	MCP-2	(C-C)
NAP-2	(C-X-C)	MCP-3	(C-C)
MGSA/gro-α	(C-X-C)	RANTES	(C-C)
GCP-2	(C-X-C)	MIP-1α	(C-C)
ENA.78	(C-X-C)	MIP-1β	(C-C)
		I-309	(C-C)
		IP-10	(C-X-C)
		PF4	(C-X-C)

Based on purified recombinant or natural material.

Table 1. Chemokines which are active on neutrophils and monocytes *in vitro*.

		T Cells	B Cells	Eosinophils	Basophils	Fibroblasts
PF4	(C-X-C)					+
β-TG	(C-X-C)					+
IL-8	(C-X-C)	±			-	
IP-10	(C-X-C)	+				
RANTES	(C-C)	+	-	+	+	
MCP-1	(C-C)	+			+	
MIP-1α	(C-C)	+	+	+	±	
MIP-1β	(C-C)	+	-	-	-	

Table 2. Chemoattractant effects *in vitro* of chemokines for cells other than monocytes and neutrophils.

interaction is transient in nature and functions to allow leukocytes to sample the local endothelium for the presence of specific 'triggering factors' that activate leukocyte integrins and allow the adhesion cascade to continue. In the absence of such factors, leukocytes disengage from the endothelium and move on. Chemokines (and other cytokines) present on the vessel wall act as triggering factors, converting the functionally inactive integrin molecules on the leukocyte to an active, adhesive configuration which promote strong adhesion to the endothelium (Tanaka *et al.* 1993). During strong adhesion, integrin mediated binding to endothelial cell counter receptors results in cessation of movement and flattening of the leukocyte. IL-8 is an important example of a neutrophil triggering factor, and is produced either by the endothelium itself, or diffuses from the perivascular tissue (Rot, 1992). IL-8 is retained on the vessel wall where it acts as an immobilised ligand for the rolling leukocyte. The C-C chemokines, MCP-1, macrophage inflammatory protein-1α (MIP-1α) and RANTES can modulate monocyte integrin expression (Vaddi & Newton, 1994), while RANTES and MIP-1β activate $CD4^+$ T cell integrins (Gilat *et al.* 1994). After strong adhesion to the endothelium, the leukocytes migrate into tissue along a chemotactic gradient. Many of the factors that trigger strong adhesion act as chemoattractants, thus IL-8 and MIP-1β induces migration of neutrophils and T cell subsets respectively, maintaining leukocyte subset specificity (Adams & Shaw, 1994). Evidence suggests migration may be a result of haptotaxis as opposed to chemotaxis (Rot, 1992), thus leukocytes may migrate along a gradient of bound substrate, as opposed to a soluble chemotactic gradient that may be diluted by flowing blood. The proteoglycan-binding properties of the chemokines may be important for their binding to endothelium and extracellular matrix. In addition to their chemoattractant capacities, certain chemokines play a role in activation of the target cell. Thus IL-8 stimulates the release of lysosomal enzymes, expression of complement receptors and increases the formation of leukotriene B_4 (LTB_4) and superoxide by neutrophils (Baggiolini *et al.* 1989).

Recent findings have indicated that chemokines may be involved in systems other than leukocyte migration. C-C chemokines have been shown to play a role in T cell development and activation, while the C-X-C chemokines are important in angiogenesis (Kunkel *et al.* 1995).

Chemokines bind to an array of shared and specific receptors on blood leukocytes. Several chemokine receptors have been cloned and all belong to the family of heterotrimeric G protein-coupled receptors, which possess seven transmembrane domains (Table 3). Two C-X-C chemokine receptors have been identified, designated C-X-C type A (or IL-8R-A) and C-X-C type B (or IL-8R-B) (Holmes *et al.* 1991; Murphy & Tiffany, 1991). C-X-C type A is specific for IL-8, while C-X-C type B is a shared receptor for the C-X-C chemokines, including IL-8, melanocyte growth stimulating activity/*gro* (MGSA/*gro*), neutrophil activating protein-2 (NAP-2) and epithelial-derived neutrophil attractant-78 (ENA-78). Receptor binding and cross-desensitisation studies indicate the existence of multiple receptors for C-C chemokines, which differ in their pattern of distribution across cell types (Sozzani *et al.* 1995). To date five C-C chemokine receptors have been identified and cloned (Table 3) (Neote *et al.* 1993; Charo *et al.* 1994; Myers *et al.* 1995; Combadiere *et al.* 1995a; Power *et al.* 1995; Hoogewerf *et al.* 1996; Combadiere *et al.* 1995b). The receptors designated C-C chemokine receptor 1 (C-C CKR-1) and C-C CKR-2 are the best characterized of these. C-C CKR-1 binds MIP-1α, RANTES, MIP-1β and MCP-1 with varying affinities, but transduces a signal in transfected kidney cells primarily in response to only MIP-1α and RANTES (Neote *et al.* 1993). C-C CKR-2 is specific for MCP-1 and two forms of the receptor exist (type A and B), which have alternatively spliced carboxy terminal tails (Charo *et al.* 1994). Furthermore, the Duffy antigen receptor for chemokines (DARC), present on human erythrocytes and on endothelial cells lining post-capillary venules in the kidney and spleen is a promiscuous receptor, which binds both C-C and C-X-C classes, as well as acting as a receptor for the malarial parasite, *Plasmodium vivax* (Hadley *et al.* 1994), The function of the Duffy antigen is not yet clear, however expression of this receptor on post-capillary endothelium may play a role in leukocyte transmigration by concentrating chemokines at the cell surface for presentation to target leukocytes, while the erythrocyte receptor may function as a 'sink' that binds and inactivates circulating chemokines.

Particular interest in the chemokine family stems from the degree of leukocyte specificity they possess, which contrasts with the non-specific action of the classical chemotactic factors such as C5a, platelet activating factor (PAF) and LTB_4. The

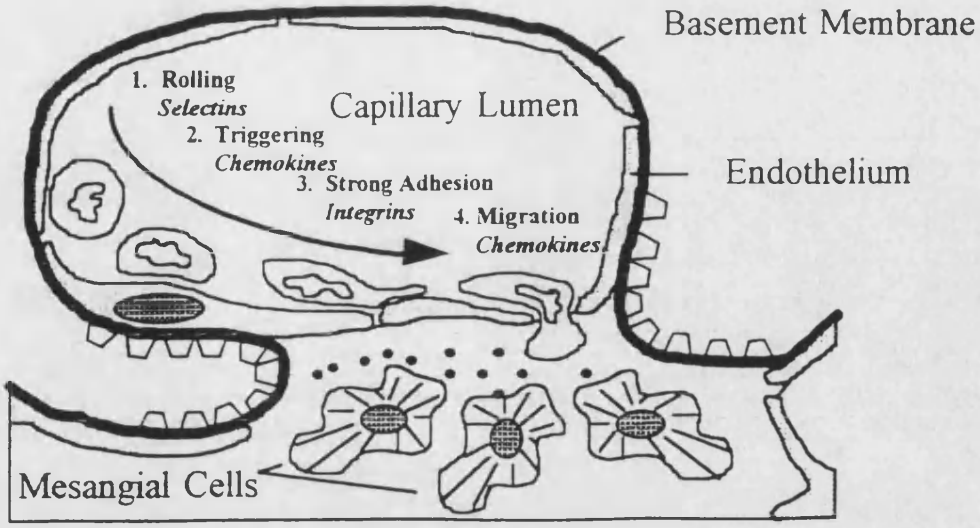


Figure 3. Schematic representation of leukocyte transmigration following activation of glomerular cells.

CHEMOKINE RECEPTORS	LIGAND SPECIFICITY
C-X-C type A	High affinity for IL-8 Low affinity for MGSA/*gro*
C-X-C type B	High affinity for IL-8, MGSA/*gro*, NAP-2, ENA-78
C-C CKR-1	High affinity for MIP-1α Low affinity for RANTES, MCP-1, MCP-3, MIP-1β
C-C CKR-2	Type A and type B: Both high affinity for MCP-1 Type B also binds MCP-3
C-C CKR-3	Binds eotaxin
C-C CKR-4	Binds MIP-1α and RANTES
C-C CKR-5	Binds MIP-1α, MIP-1β and RANTES
DARC	Promiscuous receptor, binds both C-X-C and C-C (not MIP-1α) chemokines

Table 3. C-X-C and C-C chemokine receptors.

specificity of the chemokine family may explain in part, the time-dependent accumulation of specific leukocyte populations during the course of acute and chronic inflammation. Complex interactions between the leukocyte and endothelial cell, involving the selective expression of cellular adhesion molecules may also play an important role (Adams & Shaw, 1994).

1.2.1. Role of chemokines in glomerulonephritis

A number of studies have implicated a role for chemokines in the pathogenesis of GN. Increased expression of MIP-2, MCP-1 and RANTES has been observed in glomeruli from rats with experimental mesangioproliferative and anti-GBM disease (Tam *et al.* 1996; Stahl *et al.* 1993; Rovin *et al.* 1994b; Thaiss *et al.* 1993). The enhanced chemokine expression correlated with the kinetics of leukocyte infiltration (Tam *et al.* 1996; Stahl *et al.* 1993). More direct evidence comes from a recent report showing administration of neutralising antibodies for IL-8 reduced glomerular neutrophil infiltration by 40% and completely normalised urinary levels of protein and albumin in rabbits with acute immune complex-induced GN (Wada *et al.* 1994a). Similarly, administration of anti-MIP-2 or anti-CINC (cytokine-induced neutrophil chemoattractant) antiserum to rats with anti-GBM nephritis attenuated neutrophil influx and reduced proteinuria (Feng *et al.* 1995; Wu *et al.* 1994).

Evidence of a chemokine involvement in human GN is more limited. Immunostaining of patient biopsy samples demonstrated glomerular MCP-1 expression in human inflammatory glomerulopathies, but not in glomerular disease lacking a prominent monocyte infiltrate (Rovin *et al.* 1994b). Urinary excretion of IL-8 was increased in patients with proliferative GN, and this was associated with immunohistological detection of IL-8 in diseased glomeruli and the appearance of higher numbers of leukocytes (Wada *et al.* 1994b). A role for RANTES in renal inflammation was demonstrated by J. Pattison *et al* (1994), who localised RANTES mRNA and protein in infiltrating mononuclear cells and renal tubular epithelium in renal biopsies from patients undergoing cell-mediated transplant rejection. Furthermore, increased mRNA expression for ENA-78 has been observed during cellular transplant rejection (Schmouder *et al.* 1995).

1.3. NITRIC OXIDE

The free radical nitric oxide (NO) is a labile molecule, which has been identified as having important roles in cell communication, cell defence and injury (Moncada *et al.* 1991). NO is synthesized from the terminal guanidino nitrogen of the semi-essential amino acid, L-arginine (Fig. 4). This oxidative process is catalysed by a family of enzymes termed NO synthases (NOS) (Knowles & Moncada, 1994) and results in the formation of NO and L-citrulline. All known isoforms of NOS require reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine dinucleotide (FAD), flavine mononucleotide (FMN) and tetrahydrobiopterin (BH_4) as cofactors, but vary in their dependency for Ca^{2+} and calmodulin. Three isoforms have been identified, brain NOS, endothelial NOS and macrophage NOS, which were cloned from rat cerebellum (Bredt *et al.* 1991), bovine and human vascular endothelial cells (Lamas *et al.* 1992; Janssens *et al.* 1992) and murine macrophages (Lowenstein *et al.* 1992), respectively. Brain NOS, which is also present in peripheral neural tissue, and endothelial NOS are constitutive enzymes (cNOS) which are calcium and calmodulin dependent. Brain and endothelial NOS share ~60% homology at the amino acid level, and the cloning of both bovine and human endothelial NOS indicates that isoform sequences for the enzyme are extremely well conserved between species (~94% homology). Macrophage NOS, which is ~50% homologous at the amino acid level to endothelial NOS, is calcium and calmodulin independent and is not constitutively expressed, but induced (iNOS) by certain cytokines and bacterial products (Knowles & Moncada, 1994).

Constitutive and inducible isoforms of NOS differ in their structure and regulation (Table 4) (Knowles & Moncada, 1994). Both neuronal and endothelial cNOS enzymes are controlled by Ca^{2+} mobilising agents in a very transient and highly controlled fashion. NO production by cNOS is small, nM quantities and short lasting. In marked contrast iNOS synthesizes NO in large (μM) amounts and is regulated at the transcriptional level (Morris & Billiar, 1994). NO production by iNOS is delayed by several hours following stimulation, but once induced, is active for hours and days. Inducible NOS is sensitive to inhibitors of DNA transcription and protein synthesis,

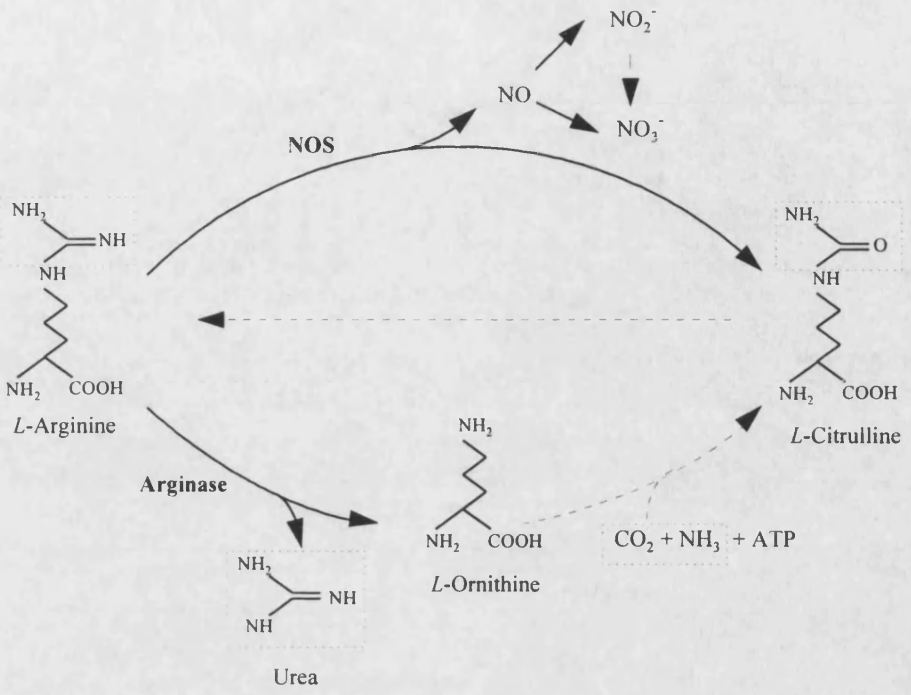


Figure 4. Pathways of L-arginine metabolism via nitric oxide synthase (NOS) and arginase (From Cattell and Cook, 1993).

Constitutive	Inducible
Both cytosolic (neuronal NOS) and particulate (endothelial NOS)	Cytosolic
Require NADPH, BH_4, FAD, FMN as cofactors	Require NADPH, BH_4, FAD, FMN as cofactors
Ca^{2+}/calmodulin dependent	Ca^{2+}/calmodulin independent
Unaffected by glucocorticoids and inhibitors of DNA transcription/translation	Inhibited by glucocorticoids and inhibitors of DNA transcription/translation
Picomoles of NO released (nM concentrations)	Nanomoles of NO released (μM concentrations)

Table 4. Comparison of constitutive and inducible NO synthase isoforms.

such as actinomycin D and cycloheximide (Radomski *et al.* 1990; Pfeilschifter & Schwarzenbach, 1990). In addition, glucocorticoids (Radomski *et al.* 1990; Pfeilschifter, 1991b) and cytokines such as TGFβ (Ding *et al.* 1990), PDGF (Pfeilschifter, 1991a), IL-8 (McCall *et al.* 1992) and IL-10 (Cunha *et al.* 1992) have been found to inhibit iNOS activity, without affecting cNOS. Constitutive NOS and iNOS also show some differences in their affinity for N^{ω}-monosubstituted arginine analogs, which compete with L-arginine for binding sites in the NO synthase enzymes and inhibit their activity (Fig. 5). Constitutive endothelial NOS showed a rank order of potency of $NO_2 > NH_2 > CH_3$ substituted analogs, in comparison to the $NH_2 > CH_3 > NO_2$ order of iNOS (Gross *et al.* 1991; Gross *et al.* 1990). Thus N^{ω}-nitro-L-arginine (L-NNA) and N^{ω}-amino-L-arginine (L-NAA) are more potent inhibitors of cNOS and iNOS respectively, while N^{ω}-monomethyl-L-arginine (L-NMMA) was found to be an effective inhibitor of both NOS isoforms. The L-arginine analogues are selective inhibitors of NOS activity, in comparison to other known inhibitors (Table 5) (Knowles & Moncada, 1994).

Expression of iNOS has been observed in a diverse range of cell types, including rat neutrophils (McCall *et al.* 1989), rat mesangial cells (Pfeilschifter & Schwarzenbach, 1990) and rat renal tubular epithelial cells (Markewitz *et al.* 1993). NOS is induced in these cells in response to a number of inflammatory stimuli, including LPS, IFN-γ, IL-1 or TNF, either alone or in combination. At the time of starting this study there was no definitive evidence for iNOS expression in human macrophages or any other human cell type. The existence of a human iNOS however, was strongly suggested by two lines of evidence. Firstly the vasopressor resistant hypotension of septic shock in humans could be improved with intravenous administration of the NOS inhibitor, L-NMMA (Petros *et al.* 1991). The role of NO as a vasodilator explains these findings. In addition, cancer patients receiving cytokine therapy had significantly elevated serum and urine nitrate levels, a stable end-product of NO synthesis (Hibbs *et al.* 1992). Metabolic tracer studies using L-[guanidino-$^{15}N_2$}arginine demonstrated the increased nitrate production was derived from the terminal guanidino nitrogen atom of L-arginine.

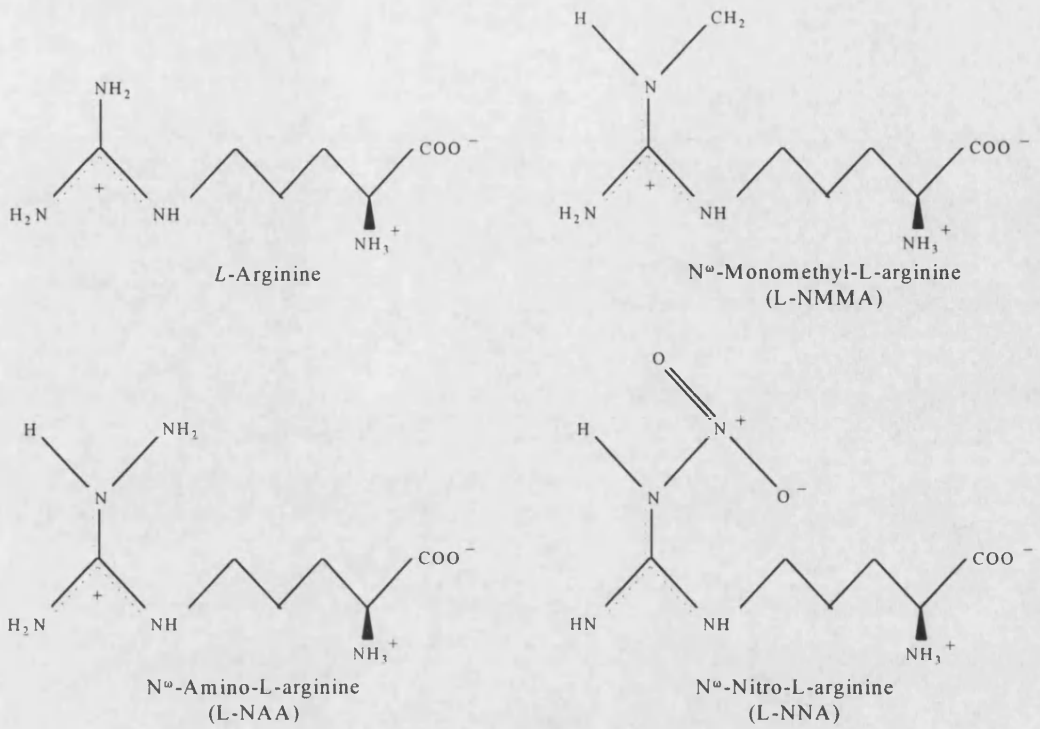


Figure 5. Structure of arginine and the arginine analogues most frequently used as NO synthase inhibitors.

Inhibitor type	Type of inhibition	Comments
Arginine analogues (e.g. L-NMMA)	Competitive with arginine	Selective
Diphenyleneiodonium	Competitive with NADPH	Also inhibits some other NADPH-enzymes
Calmodulin antagonists (e.g. trifluoperazine)	Competitive with calmodulin	Only inhibit some NO synthases; also inhibit other calmodulin-dependent enzymes
BH_4 synthesis inhibitors (e.g. DAHP, *N*-acetyl-5-hydroxytryptamine)	Indirect	
NO, CO	Not known	Probably inhibit by interacting with NO synthase haem

Table 5. Types of NO synthase inhibitors.

NO has numerous physiological and pathophysiological actions (Moncada *et al.* 1991). The major roles so far identified are in the control of blood pressure and vascular tone, neurotransmission, mediation of macrophage cytotoxicity and inhibition of platelet aggregation and adhesion to endothelium. The mechanism for these diverse effects involves the local diffusion of NO from generator to target cells, which represents a novel signal transduction mechanism between cells. Thus endothelial cNOS can be activated by vasodilators such as acetylcholine, bradykinin and ADP, or by shear stress forces, all of which result in Ca^{2+} mobilisation. The NO produced diffuses to underlying vascular smooth muscle cells and initiates relaxation by binding to the haem moiety of soluble guanylate cyclase, causing enzyme activation and elevation of the intracellular second messenger, cyclic guanine-5'-monophosphate (cGMP). Similarly, NO generated by endothelial cNOS participates in the regulation of the glomerular microcirculation by modifying the tone of the afferent arteriole and mesangial cells (Raij & Baylis, 1995). Elevation of intracellular cGMP also mediates the inhibitory effects of NO on platelet aggregation, its role as a neurotransmitter in the brain and peripheral nervous system and the ability of NO to inhibit leukocyte transmigration and adhesion (Moncada *et al.* 1991; Kubes *et al.* 1991).

NO, when released in large quantities by iNOS activity can inhibit key enzymes by nitrosylation of reactive groups, such as iron-sulphur centres (Fe-S) and thiol groups (-SH), which are essential for enzyme catalytic function (Molina Y Vedia *et al.* 1992; Cattell & Cook, 1993). NO can inhibit enzymes in the mitochondrial electron transport chain and citric acid cycle which may account for the cytotoxic and cytostatic effects of macrophage-derived NO on tumour cells and micro-organisms. In addition, the anti-proliferative effects of NO may be due to inhibition of DNA synthesis via inactivation of the ribonucleotide reductase enzyme. The cytotoxic activity of NO may play a role in tissue injury during inflammatory diseases, and the production of NO can lead to the generation of more toxic radicals through its reaction with superoxide, forming peroxynitrite, which degrades to form hydroxyl radicals (Beckman *et al.* 1990).

The diverse biological effects of NO therefore depend upon the local concentration of NO and the specific location where NO is generated.

1.3.1. Role of nitric oxide in glomerulonephritis

NO has been associated in the induction and progression of experimental GN. Studies have shown glomeruli isolated from rats with immune complex induced GN produce increased amounts of nitrite, to micromolar levels when incubated ex vivo (Cattell *et al.* 1990; Cattell *et al.* 1991). Inducible NOS mRNA expression was elevated in glomeruli from rats with accelerated NTN (Cook *et al.* 1994) and iNOS was detected by immunohistochemistry in rats with immune complex mediated disease (Jansen *et al.* 1994). In this latter study, iNOS was detected within the intraglomerular mononuclear cells, and the majority of iNOS expressing cells were found to be macrophages. Earlier studies by Cattell *et al* (1991; 1993) have suggested that infiltrating macrophages were the major source of glomerular NO during immune injury, since they demonstrated a good correlation between nitrite generation and the extent of macrophage infiltration in glomeruli from nephritic rats. In addition they found that nitrite was produced by macrophages isolated from nephritic glomeruli and that nitrite production was inhibited when macrophages were depleted *in vivo* (Cattell *et al.* 1991; Cattell & Cook, 1993). The concomitant participation of intrinsic glomerular cells to increased NO synthesis during glomerular inflammation, however cannot be excluded, since iNOS expression by rat and bovine mesangial cells in culture has been demonstrated (Pfeilschifter & Schwarzenbach, 1990; Marsden & Ballermann, 1990). The relative contribution of intrinsic glomerular cells to NO synthesis is difficult to establish since induction of NOS in these cells in culture requires cytokines such as IL-1, TNF and IFN-γ. Thus while primed macrophages may be the main source of NO early during inflammation, the release of macrophage-derived cytokines may activate intrinsic glomerular cells to synthesise cytokines and express NOS by autocrine and/or paracrine mechanisms. Macrophage infiltration may therefore be necessary to trigger iNOS expression in intrinsic glomerular cells (Pfeilschifter *et al.* 1993).

The role of NO during glomerular inflammation is unclear, since NO appears to exhibit both cytotoxic and cytoprotective effects. The cytotoxic activity of NO has been demonstrated in MRL-*lpr/lpr* mice, which develop glomerulonephritis during the course of a spontaneous autoimmune disease. Synthesis of NO is increased in these mice and the use of a NOS inhibitor (L-NMMA) attenuated the development of GN

(Weinberg *et al.* 1994). Studies in the anti-Thy-1 model of mesangioproliferative GN showed mesangial cell injury and subsequent accumulation of extracellular matrix were both reduced by 90% with NOS inhibition (Narita *et al.* 1995). In contrast, Waddington *et al* (1996) demonstrated inhibition of iNOS activity using arginase lead to increased proteinuria in the NTN model of GN in rats. In the systemic model of the disease, administration of arginase also resulted in increased glomerular thrombosis and an increase in the severity of histological changes, accompanied by systemic hypertension. These later findings suggest NO may have an important role in limiting acute glomerular injury in this model of GN.

1.4. GLOMERULAR MESANGIAL CELLS

1.4.1. Cells of the mesangium

The detailed microscopic observation of Zimmerman in 1933 first identified the mesangium as the central connective-tissue like space of the glomerular capillary tuft. The mesangium is now known to be a highly specialised, branching pericapillary tissue which is considered to possess two principal cell populations: a vascular smooth muscle type cell called the mesangial cell (MC) that represents the predominant cell type and a rare (3 to 7 % of mesangial cell population) bone-marrow derived phagocytic cell. Electron microscopy demonstrated MC to be irregular in shape, with numerous pseudopods of varying length extending into the surrounding matrix and connected to the GBM. A prominent feature of these cells is the presence of numerous bundles of small intracellular filaments and associated attachment bodies similar to those found in smooth muscle cells. A further similarity between MC and smooth muscle cells is the presence of angiotensin II receptors on the plasma membrane (Foidart *et al.* 1980). The small subpopulation of resident phagocytic cells, demonstrated in rat glomeruli, display many features of monocytes/macrophages, expressing Fc and C3b receptors, the common leukocyte antigen and many also bear Ia determinants (Striker & Striker, 1985; Kashgarian & Sterzel, 1992).

The major function of the MC within the kidney is to control the rate of glomerular filtration primarily through the regulation of mesangial cell tone. A secondary function of this specialised pericyte is to maintain the integrity of the glomerulus via matrix synthesis and provide support for the capillary loops. Furthermore, because of its close contact to the glomerular capillary lumen via the fenestrated endothelium, the mesangium is constantly perfused by macromolecules and filtration residues. While the resident macrophage type cells may play a major role in clearing macromolecules by phagocytosis, *in vitro* evidence suggests MC also have specific uptake mechanisms for macromolecules, including immune complexes. Endocytosis of immune complexes by MC in culture is mediated by surface Fc receptors, and uptake results in the generation of a variety of inflammatory mediators (Schlöndorff, 1987).

1.4.2. Inflammatory mediators released by 'activated' mesangial cells

The establishment of MC in culture has increased understanding of the biochemistry and physiology of these cells, and shown that MC can serve as both a source and target for numerous cytokines, eicosanoids and reactive oxygen intermediates (Mene *et al.* 1989; Sedor *et al.* 1993). Both *in vitro* and *in vivo* evidence indicates MC do not play a bystander role in inflammatory disorders, but instead participate in the initiation and maintenance of glomerular inflammation via the release of inflammatory mediators (Sedor *et al.* 1993).

Cultured MC provide a good model system for studying the potential of these cells to participate in inflammatory events, as these cells express a phenotype in culture which may mimic their characteristics within the inflamed glomerulus (Mene *et al.* 1989). In the normal *in vivo* situation, MC are exposed only to plasma, while in culture they are exposed to non-autologous serum that contains products produced by platelets and by activation of the coagulation system. Furthermore, the initiation of cell culture necessitates active and prolonged cellular proliferation, while intrinsic glomerular cells in the normal adult have very low replicative rates. This transition to an activated, rapidly proliferating state may however be associated with the loss or gain of certain phenotypic properties, which need to be considered when interpreting *in vitro* studies (Lovett & Sterzel, 1986).

1.4.2.1. *Mesangial cell-derived cytokines*

Cultured MC produce cytokines and growth factors which may act in both an autocrine and paracrine manner. Many of the growth factors thought to be mitogenic for MC can be produced by the cells themselves, including IL-6, PDGF and IGF-1 (Ruef *et al.* 1990; Silver *et al.* 1989; Aron *et al.* 1989). Production of these factors by MC can occur in response to each other, and themselves (Abbott *et al.* 1992; Brown *et al.* 1991a; Ruef *et al.* 1990; Silver *et al.* 1989), which complicates the interpretation of their specific effects in this system.

A number of studies have demonstrated human MC can express IL-1. IL-1α and β mRNA was detected in human MC treated with serum, PDGF or EGF (Lovett & Larsen, 1988), and IL-1β was found to dose-dependently increase its own expression in proliferating cells (Zoja *et al.* 1992). A further study showed both proliferating and quiescent human MC in culture expressed IL-1α mRNA following cytokine stimulation, and the IL-1 protein produced remained cell associated (Abbott *et al.* 1992). Reports concerning an ability of human MC to express TNF are limited, and contradictory. Schonermark *et al* (1991) describe TNF production by MC following stimulation with IL-1 or the membrane attack complex, C5b-9, while Abbot *et al* (1992) failed to detect TNF mRNA in either proliferating or quiescent MC following IL-1β, TNF or LPS stimulation.

1.4.2.2. *Mesangial cell-derived chemokines*

Glomerular MC in culture produce a number of chemokines upon appropriate stimulation. Human MC, when activated by IL-1 or TNF express and secrete IL-8 and MCP-1 (Brown *et al.* 1991b; Abbott *et al.* 1991; Kusner *et al.* 1991; Rovin *et al.* 1992; Brown *et al.* 1992). Elevated IL-8 and MCP-1 mRNA expression was detected as early as 1 hour post-cytokine stimulation (Abbott *et al.* 1991; Rovin *et al.* 1992), and quantitation of IL-8 protein production demonstrated levels were still increasing at 24 hours (Brown *et al.* 1991b). The MC-conditioned media was shown to be chemotactic for both neutrophils (Kusner *et al.* 1991) and monocytes (Rovin *et al.* 1992) *in vitro*, and leukocyte migration was inhibited by ~60% with anti-IL-8 or by ~80% with anti-MCP-1 neutralising antibodies, respectively. IL-1 was a more potent stimulant than

TNF for both IL-8 and MCP-1 production in human MC (Brown *et al.* 1991b; Brown *et al.* 1992), and the stimulatory effects of IL-1 were completely inhibited following pretreatment with IL-1ra (Brown *et al.* 1992). Furthermore, the glucocorticoid, dexamethasone was partially effective at reducing both IL-1 and TNF induced IL-8 production (Brown *et al.* 1991b).

Increased MCP-1 production has also been observed in IFN-γ or thrombin stimulated human MC (Grandaliano *et al.* 1994b; Grandaliano *et al.* 1994a), and by mouse MC stimulated with either IFN-γ, TNF or IgG immune complexes (Satriano *et al.* 1993). In addition, a recent study performed in a murine mesangial cell line describes RANTES expression and production following stimulation with TNF, IL-1 or LPS (Wolf *et al.* 1993).

Expression of MCP-1 in glomeruli has been demonstrated in the anti-Thy-1 rat model of GN (Stahl *et al.* 1993). Immunohistologic staining localised MCP-1 production to the mesangium. The correlation between the timing of increases and decreases in glomerular MCP-1 expression, with that of mesangioproliferation and mesangiolysis during progression of the disease indicated MC may be a major source of MCP-1 in this model, not the infiltrating monocytes/macrophages.

1.4.2.3. *Mesangial cell-derived prostaglandins*

Eicosanoids are metabolites of arachidonic acid, a polyunsaturated long chain fatty acid which is stored in cell walls esterified in phospholipids. Arachidonic acid is the substrate for both cyclooxygenase and lipoxygenase, and multiple eicosanoid products are derived from these two pathways (Fig. 6). Eicosanoids have a wide range of biological activities, which contribute to the cellular response to injury (Rola-Pleszczynski, 1985; Goodwin & Webb, 1980; Klahr & Harris, 1989). Thromboxane A_2 (TXA_2) can cause platelet aggregation and granulocyte adhesion, as well as acting as a vasoconstrictor, while the prostaglandins, PGE_2 and PGI_2 are vasodilatory. The peptido-leukotrienes LTC_4, LTD_4 and LTE_4 are vasoconstrictors, LTB_4 in comparison has wider proinflammatory and immunoregulatory effects, functioning as a

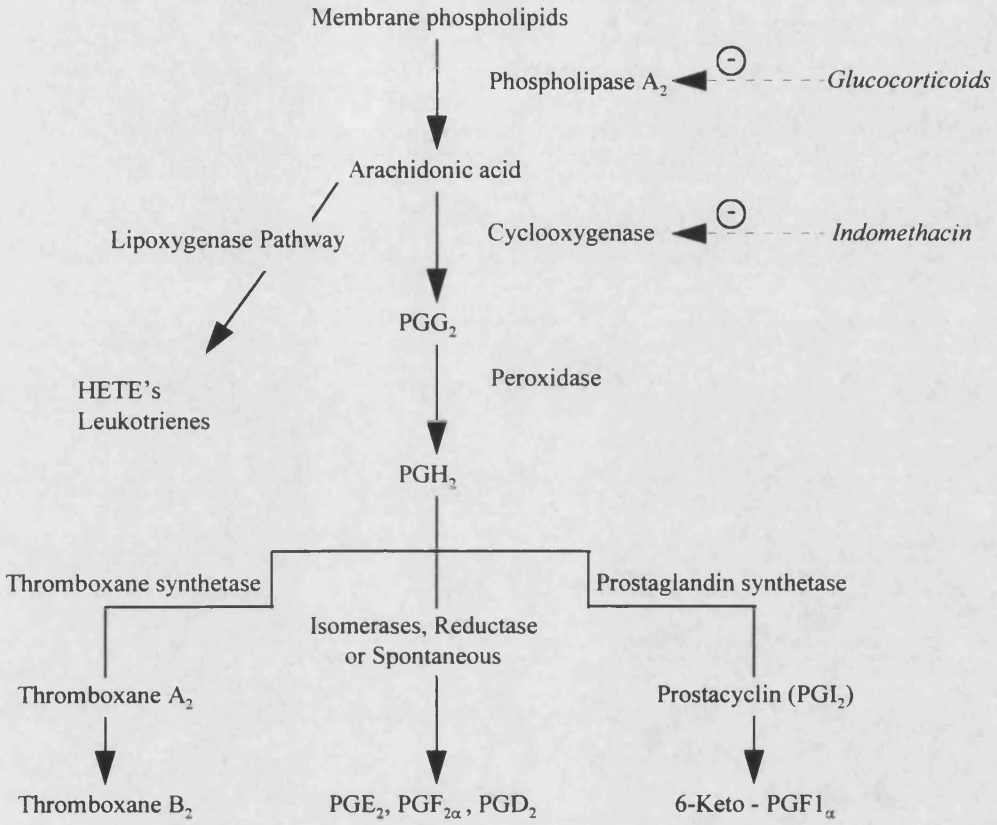


Figure 6. Biosynthesis of prostaglandins and thromboxane. The broken lines indicate the site of action of known inhibitors of this pathway (Abbreviations; HETE=hydroxyeicosatetraenoic acid, PG=prostaglandin)

chemoattractant for leukocytes and inducing other inflammatory mediators, including cytokines (Rola-Pleszczynski & Stankova, 1992).

Human mesangial cells in culture were found to constitutively release trace amounts of the prostaglandins, PGE_2, PGF_{2_α} and 6-keto-PGF_{1_α}, the latter being the stable end product of prostacyclin (PGI_2) formation (Mene *et al.* 1989). Both IL-1 and TNF increase prostaglandin synthesis by cultured human MC, with PGE_2 and 6-keto-PGF_{1_α} being the major prostaglandins produced (Topley *et al.* 1989). Both these cytokines induce the expression and activation of secretory and cytosolic forms of the phospholipase A_2 (PLA_2) enzyme in rat MC, which results in the release of arachidonic acid from membrane phospholipids (Schalkwijk *et al.* 1991; Schalkwijk *et al.* 1992). These cytokines also induce *de novo* expression of the cyclooxygenase enzyme (Topley *et al.* 1989). Increased production of vasodilatory prostaglandins may have a protective effect on renal function during chronic renal disease by maintaining glomerular filtration rates (Klahr & Harris, 1989). Prostaglandins may also influence local cell proliferation, synthesis and breakdown of extracellular matrix and cytokine production (Schlöndorff, 1987). *In vitro*, PGE_2 downregulates TNF production by endotoxin stimulated monocytes/macrophages via elevation of the intracellular second messenger cyclic adenosine-5'-monophosphate (cAMP) (Schade & Schudt, 1993).

1.4.2.4. *Production of oxygen radicals by mesangial cells*

Release of the oxygen radical, superoxide (O_2^-) has been mainly described as a specific function of phagocytic leukocytes, where it is required for the killing of bacteria and parasites (Jones *et al.* 1993). However, recent studies have shown human mesangial cells also possess a similar plasma membrane-bound NADPH-dependent oxidase, which catalyses O_2^- production (Radeke *et al.* 1991; Jones *et al.* 1993). O_2^- release has been demonstrated in culture human MC following stimulation with IL-1, TNF and the calcium ionophore A23187 (Radeke *et al.* 1990). In comparison to neutrophils and eosinophils, the rate of O_2^- release by MC was low (nanomoles/hour) and did not appear to be toxic to the MC.

O_2^- generation results in spontaneous or catalysed formation of other reactive oxygen intermediates which differ in their toxicity to cells (Fig. 7). Lipids, proteins, nucleic acids and carbohydrates are all major targets for oxidative modification that can result in tissue injury (Johnson *et al.* 1994). While MC may contribute to the oxygen-radical induced damage during GN, together with infiltrating leukocytes, recent reports have suggested production of lower concentration of oxygen radicals by non-immune cells (e.g. MC, fibroblasts and endothelial cells) may have important regulatory functions, in particular in cellular signalling (Jones *et al.* 1993). A role for O_2^- as a second messenger for MCP-1 and RANTES production in cytokine activated mouse MC has recently been suggested (Satriano & Schlondorff, 1994a; Satriano & Schlondorff, 1994b).

1.4.2.5. *Production of nitric oxide by mesangial cells*

Induction of NO synthase activity in cultured rat and bovine glomerular MC was first described in 1990, following stimulation with IL-1, TNF or LPS (Pfeilschifter & Schwarzenbach, 1990; Marsden & Ballermann, 1990; Shultz *et al.* 1991). Due to the labile properties of NO, its production was demonstrated by measuring elevated intracellular cGMP levels and the formation of micromolar amounts of extracellular nitrite, a stable end-product of NO degradation. The induction of cGMP formation required a lag phase of 4-8 hours and was inhibited by actinomycin D and cycloheximide, indicating *de novo* protein synthesis was required (Pfeilschifter & Schwarzenbach, 1990; Marsden & Ballermann, 1990). The MC derived NOS was characterized as being of the macrophage inducible type, as the L-arginine analogue, L-NMMA was more potent than L-NNA in inhibiting cytokine induced cGMP formation and enzyme activity appeared independent of Ca^{2+} and calmodulin (Pfeilschifter *et al.* 1992). Cytokine induction of NOS activity in rat MC was dose-dependently inhibited by TGFβ (Pfeilschifter & Vosbeck, 1991), glucocorticoids (Pfeilschifter, 1991b) and PDGF (Pfeilschifter, 1991a). Furthermore, cAMP-elevating agents alone induced iNOS expression and activity above basal levels and when added in combination with IL-1, enhanced nitrite production in a synergistic manner (Kunz *et al.* 1994).

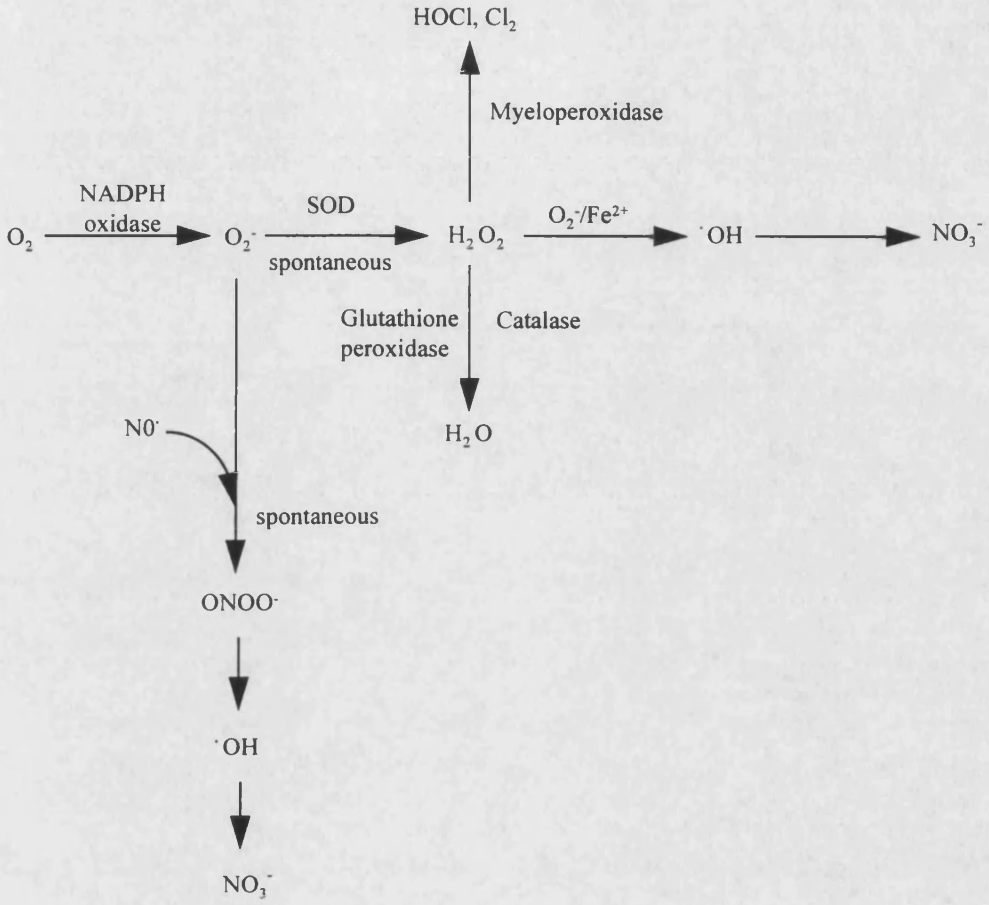


Figure 7. Formation of other free radicals from superoxide. Superoxide anions (O_2^-) are produced by the univalent reduction of oxygen by NADPH oxidase. Dismutation of O_2^-, either spontaneously or catalysed by superoxide dismutase (SOD) yields hydrogen peroxide (H_2O_2). The H_2O_2 can react with myeloperoxidase and chloride to form hypochlorous acid (HOCl) and chlorine (Cl_2), or it can react with iron and O_2^- to form hydroxyl radicals ($^{\cdot}OH$) (Haber-Weiss reaction). Protection from the toxic effects of H_2O_2 is afforded by catalase and glutathione peroxidase, which degrade H_2O_2 to water.

In the presence of nitric oxide ($NO^{\cdot}$), O_2^- is converted to peroxynitrite ($ONOO^-$), which also leads to $^{\cdot}OH$ generation. The stable end product of $^{\cdot}OH$ formation is nitrate (NO_3^-).

1.5. POTENTIAL SIGNAL TRANSDUCTION MECHANISMS FOR IL-1 OR TNF ACTIVATION OF MESANGIAL CELLS

In vitro studies indicate both IL-1 and TNF are important in 'activating' MC to express a phenotype that contributes to the progression of glomerular inflammation through the differential induction of specific genes that can result in altered matrix metabolism, an altered cell growth response and secretion of cytokines and inflammatory mediators (Sedor *et al.* 1993). Such changes at the cellular level culminate in the histological and functional characteristics of renal injury. The molecular mechanisms by which IL-1 and TNF activate cells remain unclear and is a controversial area of signal transduction since virtually all known signalling systems have been implicated (O'Neill, 1995; Saklatvala & Guesdon, 1992). Increasing evidence suggests the IL-1 and TNF signalling mechanisms may be cell-specific, and depend upon the particular cellular response under investigation (O'Neill, 1995)

1.5.1. IL-1 receptors

Two distinct plasma membrane receptors have been identified for IL-1. The type I receptor (80 kDa) is the predominant form, expressed on MC, T lymphocytes, fibroblasts, and endothelial cells, while the type II receptor (60 kDa) occurs mainly on B cells, monocytes, neutrophils and hepatoma cells (O'Neill, 1995; Zoja *et al.* 1992). The type I receptor binds IL-1α, IL-1β and IL-1ra with equal affinity, while the type II receptor bind IL-1β with greater avidity than IL-1α or IL-1ra (Arend, 1991). Both types of IL-1 receptor have been cloned from mouse and human cell lines (McMahan *et al.* 1991) and share 28% homology at the amino acid level in the extracellular domain. The receptors possess a single membrane spanning domain and belong to the immunoglobulin supergene family, to which many growth factor and cytokine receptors belong (Taga & Kishimoto, 1992). The cytoplasmic region of the type II IL-1 receptor comprises of only 29 amino acids, in comparison to 215 amino acids in this region of the type I receptor, and evidence based on antibody blocking studies (Stylianou *et al.* 1992; Sims *et al.* 1993) strongly suggests the type II receptor is not involved in IL-1 signalling. The type II receptor may function as an extracellular IL-1 inhibitor as it has been shown to be shed from cells, thus it may bind IL-1, preventing it from interacting

with the type I receptor (O'Neill, 1995). The main features of the type I receptor is its high affinity for IL-1 and the relatively low number of receptors per cell (200-4000 receptors/cell) (Saklatvala & Guesdon, 1992). Intriguingly, receptor occupancy studies have indicated less than 10 IL-1 receptors per cell need to be occupied to elicit a biological response (Stylianou *et al.* 1992; Curtis *et al.* 1989).

1.5.2. TNF receptors

Two distinct cell surface receptors for TNF have been identified, termed TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2) (Tartaglia & Goeddel, 1992). These receptors are ~55 kDa and ~75 kDa respectively and both are expressed on most cell types, with the number varying between 100 and 10,000 per cell (Beyaert & Fiers, 1994). Both receptors have a single, membrane spanning domain and are 28% homologous in the extracellular region. The TNF receptors are members of a growing superfamily of receptors that includes the fas antigen, CD40 and gp50, all of which possess four extracellular repeats of a cysteine rich domain (Smith *et al.* 1994). The intracellular domains of TNF-R1 and TNF-R2 consist of 221 and 174 amino acids respectively, and show no detectable similarity in their amino acid sequences which suggests they utilise distinct signalling pathway and mediate distinct TNF activities (Lewis *et al.* 1991). This latter suggestion has been confirmed in gene-knockout experiments and studies using antibodies that act as specific agonists or antagonists of the two receptor types (Pfeffer *et al.* 1993; Engelmann *et al.* 1990; Wong *et al.* 1992; Tartaglia *et al.* 1993; Kruppa *et al.* 1992). The majority of TNF activities appear to be mediated by TNF-R1 including cytotoxicity, cytokine induction, activation of the transcription factor nuclear factor κB (NF-κB) and prostaglandin synthesis (Engelmann *et al.* 1990; Wong *et al.* 1992; Kruppa *et al.* 1992; Tartaglia *et al.* 1993). Direct signalling through TNF-R2 appears to be mainly confined to T lymphocytes, where it is involved in cytokine expression and/or proliferation. However, a model of receptor co-operation has been proposed, where the R2 facilitates binding of TNF to R1 at low concentrations of TNF, since TNF has a higher affinity for R2 than R1 (Tartaglia & Goeddel, 1992). Studies on TNF-R1 suggest that crosslinking of the receptors is important in the activation process. The native forms of TNF and lymphotoxin have been described as trimers and thus may exert their effects by binding to and clustering the TNF receptors (Engelmann *et al.*

1990; Tartaglia & Goeddel, 1992). Both forms of the TNF receptor may also be shed from the cell, which may act to inhibit TNF activity. However, the effect of soluble TNF receptors on TNF function needs to be further explored, since the binding of TNF in a dissociable form may effect the pharmokinetics and stability of the TNF molecule (Fiers, 1991).

1.5.3. Heterotrimeric G proteins

Heterotrimeric G proteins are guanine nucleotide binding proteins which couple receptors to effector enzymes and many classes of ion channels (Hepler & Gilman, 1992). G proteins provide a primary means of amplifying a signal, as a single occupied receptor can activate many G proteins, which in turn can activate more than one of the effectors. Activation of effector enzymes such as adenylyl cyclase or phospholipase C (PLC) leads to the generation of second messengers, that ultimately leads to cellular responses.

G proteins consist of 3 subunits, α, β and γ, which are complexed in their inactive state, with GDP bound to the α subunit (Fig. 8). Receptor activation induces a GDP-GTP exchange, upon which the α subunit dissociates from the $\beta\gamma$ dimer to interact with the appropriate effector system. The cycle is complete, when GTP is hydrolysed by the intrinsic GTPase activity of the α-subunit and reassociation with the $\beta\gamma$ dimer occurs (Hepler & Gilman, 1992). Originally, the α subunit was thought to be solely responsible for downstream regulation of effector systems, while the $\beta\gamma$ dimer functioned as a helper protein which facilitated both the activation and deactivation of the α subunit, and its localisation to the membrane. However, recent studies have demonstrated the $\beta\gamma$ dimer can also directly regulate some downstream events, in addition to the predominant role of the α-subunits (Clapham & Neer, 1993). A role for $\beta\gamma$ dimers in the regulation of the β-sub-family of PLC enzymes and adenylyl cyclase has been established (Clapham & Neer, 1993; Ueda & Tang, 1993). To date, at least 20 different α-subunits have been identified in mammals, along with 5 different β-subunits and 12 γ-subunits (Neer, 1995). The α-subunits confer the specificity of effector interaction (Table 6) and individual cells usually contain at least 4 or 5 types of α subunit. Differences between the α-subunits are highlighted by their susceptibility to

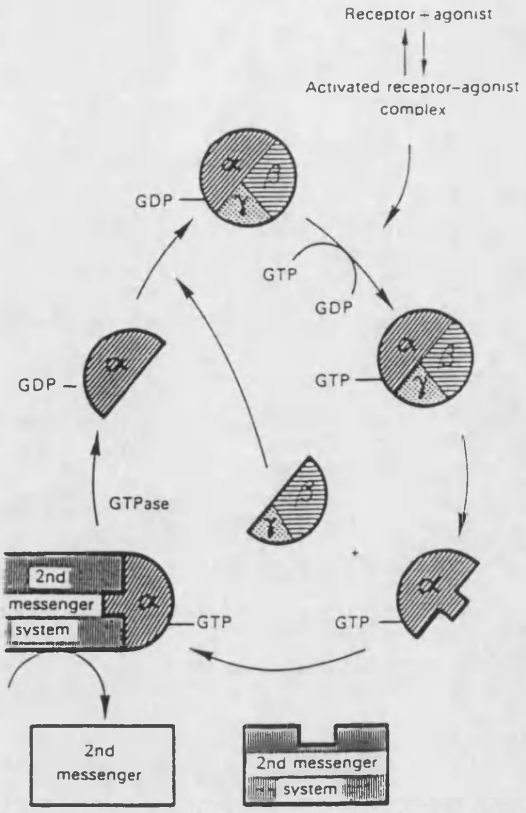


Figure 8. The role of GTP binding and hydrolysis in the activation and deactivation of a typical G protein. (From Milligan *et al*, 1988)

Class	Members	MW	Tissue distribution	Function	Modifying toxin
G_s	α_s	45 to 52	Ubiquitous	Stimulate adenylyl cyclase, regulate Ca^{2+} channels	CT
G_i	α_{i1}	41	Restricted 1° to neural tissue	Inhibit adenylyl cyclase, regulate K^+ and Ca^{2+} channels, activate PLC	PT
	α_{i2}	40	Ubiquitous		
	α_{i3}	41	Fairly ubiquitous		
	α_{o1} , α_{o2}	39	Major brain G protein, some cells		
G_q	α_q , α_{11} , α_{14} , α_{15} , α_{16}	42	Fairly ubiquitous	Mediate PT-insensitive activation of PLCβ isoforms	-
G_{12}	α_{12} , α_{13}	44	Ubiquitous	Regulate Na^+/K^+ exchange	-

Table 6. Properties of the more common G protein sub-types. Apparent molecular weights are shown. Two long forms and two short forms of $G_{\alpha s}$ exist, which are splice variants of a single gene. The two forms of $G_{\alpha o}$ are also splice variants .

modification by the bacterial toxins, cholera toxin (CT) and pertussis toxin (PT) (Milligan, 1988). These toxins are typical A-B toxins, consisting of 5 different polypeptide subunits held together by non-covalent interactions. The A subunit (S1 subunit) catalyses the transfer of ADP-ribose from NAD^+ to the α-subunit of relevant G protein substrates, with important functional consequences. The B portion of the toxin (B oligomer) consists of the subunits S2 to S5, which are arranged as two dimers, S2-S4 and S3-S4, linked by S5. It functions to bind the toxin to specific cell surface receptors and deliver the S1 subunit through the target cell membrane (Kaslow & Burns, 1992). CT ADP-ribosylates the stimulatory G protein, G_s, which leads to its permanent activation through disruption of the intrinsic GTPase activity of the α_s subunit. PT ADP-ribosylates a distinct group of G proteins, including all the isoforms of G_i and G_o, leading to their inactivation by preventing receptor-G protein coupling (Milligan, 1988). The bacterial toxins have thus proved to be useful tools for investigating signal transduction pathways. If the toxins inhibit or stimulate a biological response to a hormone, $G_{i/o}$ or G_s respectively can be implicated in the signal transduction mechanism.

The sites of cholera and pertussis toxin modification in susceptible α-subunits are different. PT ADP-ribosylates a conserved cysteine residue located 4 amino acids from the C-terminus (Milligan, 1988). This region of the subunit is thought to be important for receptor interaction, which is disrupted by ADP-ribosylation, leading to inhibition of G protein function. α subunits that are not substrate for PT lack this cysteine residue in the C-terminus (Milligan, 1988). In contrast, CT modifies an arginine residue which is postulated to be located close to the guanine-nucleotide binding domain in the tertiary structure of the α-subunit. Interestingly, the position of this arginine residue and the surrounding region is highly conserved in all G proteins, yet very few serve as substrates for CT. It has been proposed that in the non-susceptible G proteins, occupancy of the site by a guanine nucleotide may hinder access of CT (Milligan, 1988). This is supported by the observation that PT substrates only become substrate for CT in the absence of guanine-nucleotides (Milligan, 1987). A new strategy for identifying PT-sensitive G protein coupled receptors has stemmed from these observations. In membrane preparations, in the absence of guanine nucleotides,

receptors which couple to PT-sensitive G proteins produce a specific enhancement of CT catalysed ADP-ribosylation of $G_{i/o}$ type proteins (Milligan *et al.* 1991; Gierschik & Jakobs, 1987; Iiri *et al.* 1989). This is thought to be due to agonist induced dissociation of GDP from the guanine-nucleotide binding domain of the receptor-linked G protein, which in the absence of guanine nucleotides remains empty and becomes susceptible to CT modification (Milligan *et al.* 1991).

A distinct family of receptors which possess the common structural feature of 7 membrane spanning domains are known to couple to heterotrimeric G proteins (Taga & Kishimoto, 1992). However, a number of studies suggest receptors which do not possess this configuration may also be G protein coupled, including the receptors for IL-1, TNF, colony stimulating factor-1 (CSF-1), IL-2, EGF and TGFβ (O'Neill *et al.* 1990a; Imamura *et al.* 1988; Imamura & Kufe, 1988; Evans *et al.* 1987; Nair *et al.* 1989; Howe *et al.* 1990). Much of the evidence indicating an involvement of $G_{i/o}$ type proteins in IL-1 and TNF signal transduction mechanisms has arisen from studies using PT, which was found to inhibit a wide range of IL-1, and some TNF post-receptor responses (Dobson *et al.* 1989; Chedid *et al.* 1989; O'Neill *et al.* 1990a; Rollins *et al.* 1991; Imamura *et al.* 1988; Clark *et al.* 1988; Brett *et al.* 1989). PT inhibited IL-1 induced prostaglandin release from fibroblasts (Chedid *et al.* 1989), IL-1 induced IL-2 expression in EL4 cells (murine thymoma cell line) (O'Neill *et al.* 1990a; Rollins *et al.* 1991) and TNF induced PLA_2 activity in endothelial cells (Clark *et al.* 1988). Interestingly however, not all IL-1 responses were found to be sensitive to PT, with IL-1 induced NF-κB activation, collagenase production and phosphorylation of the EGF receptor being unaffected by toxin treatment (O'Neill *et al.* 1992). Additional evidence of a role for G proteins in IL-1 and TNF signalling mechanisms has come from studies showing both these cytokines specifically increase GTPase activity and GTP binding to cell membrane preparations (O'Neill *et al.* 1990a; Chedid *et al.* 1989; Imamura *et al.* 1988). The ability of PT to inhibit the stimulating effects of IL-1 and TNF in these assays further indicates that the IL-1 and TNF receptors are coupling to PT-sensitive G proteins, such as G_i or G_o isoforms.

1.5.4. Second messengers and protein kinases

Evidence obtained in human fibroblasts, and also in MC, shows IL-1 and TNF, upon receptor binding, stimulate a rapid increase in the phosphorylation of a number of cellular proteins, chiefly on serine and threonine residues, indicating the activation of kinases and/or inhibition of protein phosphatases specific for these target residues (Guy *et al.* 1991; Guy *et al.* 1993; Saklatvala & Guesdon, 1992; Lovett *et al.* 1988). The identity of the kinases or phosphates involved remain to be identified (O'Neill, 1995). Earlier work investigated the role of known serine/threonine protein kinases, such as protein kinase A (PKA) and protein kinase C (PKC) in IL-1 and TNF signalling pathways. The findings however, were controversial with some studies implicating an involvement of these kinases in IL-1 and TNF signalling events (Mizel, 1990; Zhang *et al.* 1988b; Baud *et al.* 1988) while others ruled out any such role (Sedor *et al.* 1992; O'Neill *et al.* 1990b; Guy *et al.* 1991). Recent studies have focused on a role for protein phosphatases and more novel second messengers and kinases. The sphingomyelin-ceramide pathway (Kim *et al.* 1991; Schütze *et al.* 1992; Ballou *et al.* 1992), the mitogen-activated protein (MAP) kinase pathway (Saklatvala *et al.* 1993) and okadaic acid-sensitive phosphatases, such as PP1 and PP2A (Guy *et al.* 1992; Guy *et al.* 1993) have all been implicated. Furthermore novel kinases, for which the substrate and/or upstream regulators have yet to be determined, may play an important role (Freshney *et al.* 1994; Guesdon *et al.* 1993; O'Neill, 1995).

1.5.4.1. *The cAMP pathway*

The ability of IL-1 to activate the adenylyl cyclase / cAMP signal transduction pathway (Fig. 9) has been investigated in some detail. IL-1 has been shown to directly induce a transient elevation of intracellular cAMP levels in a variety of cell types (Mizel, 1990; Chedid *et al.* 1989; Zhang *et al.* 1988b; Schirakawa *et al.* 1988). Furthermore, pharmacological agents which elevate intracellular cAMP by a number of different mechanisms (Fig. 9) have been shown to mimic several actions of IL-1. cAMP analogues and forskolin were found to replace IL-1 in inducing PLA_2 secretion in rat MC (Pfeilschifter *et al.* 1991), IL-2 receptor expression on YT lymphocytes (human natural killer-like T cell line) (Schirakawa *et al.* 1988), κIg light chain expression in the pre-B-cell line, 70Z/3 (Chedid *et al.* 1989) and production of IL-6 in human fibroblasts

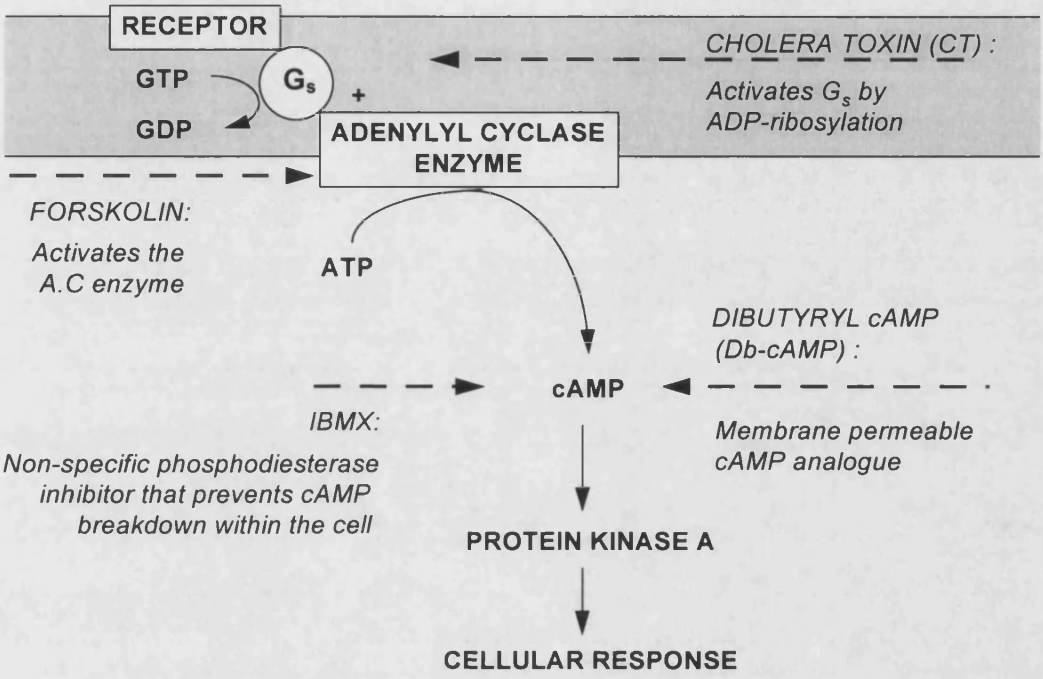


Figure 9. The cAMP signal transduction pathway: Site of action of cAMP-elevating agents. Agonist-bound receptor activation of the stimulatory G protein, G_s leads to activation of adenylyl cyclase, the enzyme responsible for the conversion of ATP to cAMP. Cellular responses are brought about by phosphorylation events involving the cAMP-dependent protein kinase (PKA), which is activated by the raised cAMP levels.

(Zhang *et al.* 1988b). In marked contrast, other investigators found no increase in cAMP levels following IL-1 activation of cells of both lymphoid and non-lymphoid origin (Ray *et al.* 1992; Bomsztyk *et al.* 1990; Rollins *et al.* 1991; Schlegel-Haueter & Aebischer, 1990), including rat MC (Pfeilschifter *et al.* 1991), and report an inability of cAMP to mimic IL-1 effects (Ray *et al.* 1992; Bomsztyk *et al.* 1990; Rollins *et al.* 1991; Schlegel-Haueter & Aebischer, 1990). Furthermore the characteristic increase in protein phosphorylation observed after IL-1 stimulation was found to occur independently of PKA activity (Guy *et al.* 1991). The reason for these differences at present are unclear, however taken together, the findings indicate cAMP is unlikely to be a major second messenger in IL-1 signalling events.

Less work has been done on the role of cAMP as a second messenger for TNF signalling, but the findings have been similarly contradictory, with some reports implicating an involvement of cAMP in mediating TNF effects (Baud *et al.* 1988; Zhang *et al.* 1988b), while others rule out any such role (Pfeilschifter *et al.* 1991; Guy *et al.* 1991).

Interestingly, a number of studies have indicated cAMP can have important regulatory effects on IL-1 activity. Elevation of intracellular cAMP by PGE_2 or pharmacological agents was found to down regulate IL-1 induced IL-2 production in EL4 cells (Rollins *et al.* 1991) and have a dual modulatory effect on IL-1 induced serine esterase activity in PC60 cells (cytotoxic T cell hybridoma) (Schlegel-Haueter & Aebischer, 1990). More recently, cAMP has been shown to downregulate IL-6 production in IL-1 stimulated lung fibroblasts (Zitnik *et al.* 1993) and downregulate IL-1 and TNF induced MCP-1 production in MC (Rovin & Tan, 1994; Satriano *et al.* 1993).

1.5.4.2. *The nitric oxide signalling pathways*

NO functions as an important signalling molecule through it's activation of soluble guanylate cyclase, resulting in elevation of the intracellular second messenger cGMP and subsequent activation of the cGMP-dependent protein kinase. An involvement of this signalling pathway in mediating smooth muscle cell relaxation and neurotransmission are well recognised (Moncada *et al.* 1991).

Recent studies have indicated a role for NO in regulating the production of certain inflammatory mediators. Brown *et al* (1993b) have demonstrated that IL-1 induced production of NO potentiates the secretion of IL-8 in human MC via elevation of intracellular cGMP. The NOS inhibitor L-NMMA, L-arginine depletion and known inhibitors of guanylate cyclase were all found to inhibit IL-1 induced IL-8 production by MC, while the NO donor sodium nitroprusside and cGMP analogues were found to enhance IL-8 generation in arginine-depleted cells. NO donors have also been reported to cause significant upregulation of both IL-8 promoter activity and IL-8 protein secretion in the human melanoma cell line, G361 (Andrew *et al.* 1995). The mechanism of NO upregulation of IL-8 gene expression involved, at least in part, activation of the transcription factor NF-κB. In addition, the ability of the NOS inhibitor N^{ω}-amino-L-arginine to reduce TNF stimulated IL-8 promoter activity by >50% indicates NO may function as an endogenous regulator of IL-8 expression in G361 cells. Endogenous production of NO in LPS and IFN-γ stimulated rat kupffer cells was found to inhibit subsequent PGE_2, TXB_2, and IL-6 synthesis by these cells, while IL-1 and TNF production was unaffected (Stadler *et al.* 1993). Furthermore, exogenous addition of NO increased TNF mRNA expression in HL-60 cells and differentially regulated IL-3 and IL-2 production by spleen cells from contact-sensitised mice (Marcinkiewicz & Chain, 1993). The role of cGMP in mediating the regulatory effects of NO on cytokine and eicosanoid production in these latter studies was not determined.

An ability of NO to activate a number of signalling systems via cGMP-independent mechanisms has been reported in human peripheral mononuclear cells. Such effects of NO include activation of the transcription factor NF-κB (Lander *et al.* 1993a) and direct activation of the heterotrimeric G proteins, G_s, G_{i1} and the small molecular weight G protein, $p21^{ras}$ (Lander *et al.* 1993b). The ability of NO to directly activate G proteins is thought to play a role in its activation of NF-κB, however the mechanism of G protein activation by NO remains unknown (Lander *et al.* 1993b). Interestingly NO increases the activity of a cytosolic ADP-ribosyltransferase in human platelets and different rat tissues by a cGMP independent mechanism (Brune & Lapetina, 1989). NO activation of this enzyme resulted in the ADP-ribosylation of a 39 kDa protein in all the tissues analysed. However, the protein was not recognised by $G_{common\ \alpha}$ antiserum, indicating

the protein is not one of the known heterotrimeric G proteins which have similar molecular masses. The importance of the NO induced ADP-ribosylation of the 39 kDa protein therefore remains to be elucidated. In addition, NO has been shown to modulate protein tyrosine kinase and protein tyrosine phosphatase activity in lymphocytes (Lander *et al.* 1993a).

The ability of NO to activate a number of different signalling pathways indicates an expanding role for this molecule in signal transduction mechanisms.

1.5.5. Transcription factors

Transcription factors are DNA binding proteins that mediate changes in gene expression. They provide the link between post-receptor signalling events and changes in transcription. Phosphorylation plays a key role in regulating their activities, and upon activation the sequence specific transcription factors bind to particular sites in the promoter region of genes and control their transcription. Different families of transcription factors have been defined, based upon their protein structure (Karin & Smeal, 1992; Peterson & Tupy, 1994).

IL-1 and TNF have been shown to activate several transcription factors, including NF-κB, NF-IL6 and AP-1 (O'Neill, 1995; Osborn *et al.* 1989; Mukaida *et al.* 1990; Yasumoto *et al.* 1992; Brown *et al.* 1993a). The activation of NFκB by IL-1 and TNF appears to be important in mediating a number of their inflammatory effects, including induction of IL-8, IL-6 and *gro* gene expression (Mukaida *et al.* 1990; Zhang *et al.* 1990; Anisowicz *et al.* 1991). The predominant form of NF-κB exists in resting cells in the cytosol as a heterodimer of p50 and p65 (relA) polypeptides complexed to an inhibitory protein, IκB. Activation of NF-κB requires dissociation from IκB, which allows translocation of the heterodimer to the nucleus, while IκB is rapidly degraded. The mechanisms that induce dissociation are poorly understood, but may involve phosphorylation of IκB (Beg & Baldwin, Jr. 1993). The kinase(s) involved however remain to be identified. PKA, PKC and the MAP kinases have all been found to activate NFκB *in vitro* (Shirakawa & Mizel, 1989).

1.6. AIMS OF THE STUDY

This study is based on the hypothesis that MC play a direct role in the initiation and propagation of inflammatory events within the glomerulus via the release of inflammatory mediators. Recent studies have demonstrated that human MC *in vitro* are a rich source of the chemokines IL-8 and MCP-1, and the mesangioproliferative cytokine IL-6 following stimulation with the proinflammatory cytokines IL-1 or TNF (Brown *et al.* 1991b; Abbott *et al.* 1991; Rovin *et al.* 1992).

The objectives of this investigation were therefore;

(1) To investigate the potential of human MC to express other members of the chemokine superfamily.

(2) To determine the molecular mechanisms that regulate the expression and release of chemokines and IL-6 in cytokine activated human MC.

(3) To identify the role of the cAMP signal transduction pathway in the regulation of IL-8 and IL-6 production in IL-1 or TNF stimulated MC.

(4) To investigate the role of pertussis toxin-sensitive heterotrimeric G proteins in the IL-1 signalling mechanisms for IL-8 and IL-6 in MC.

(5) To define the potential role of the L-arginine/nitric oxide signal transduction pathway in IL-1 induced IL-8 generation in MC.

Increasing our understanding of the signal transduction pathways that regulate chemokine and IL-6 production in human MC may provide leads for the development of novel and effective therapeutic strategies for the prevention of glomerular inflammation.

2. MATERIALS AND METHODS

Recipes for the buffers used in the following method sections, and specific details relating to the storage and handling of drug solutions are contained in appendices I-VI at the end of the thesis.

2.1. MESANGIAL CELL ISOLATION AND CULTURE CONDITIONS

Details of the buffers and solutions used for cell culture are given in appendix II. Sterile plastics and pastettes were purchased from Biddy Sterilin Ltd (Store, UK) and Scientific Laboratory supplies (Nottingham, UK), respectively. All reagents and equipment which were not purchased sterile were autoclaved at 20 lb/in^2 for 20 min at 121°C before use. Glassware was autoclaved at 30 lb/in^2 for 30 minutes at 135°C.

All tissue culture plastics (Nunc, Denmark) were coated with 1% gelatin for 1 hour at 37°C, then washed 1x with PBS, prior to the addition of cells.

2.1.1. Mesangial cell isolation

Human mesangial cells (MC) were isolated from macroscopically normal portions of renal cortex, obtained from human kidneys immediately after surgical nephrectomy for renal carcinoma. Tissue was provided by Dr C. Gallagus (Royal United Hospital, Bath) and was collected in 50 ml sterile Hanks balanced salt solution (HBSS), pH 7.4. MC were isolated according to the method described by Striker and Striker (1985) and Lovett *et al* (1983). Glomeruli were isolated from renal cortex by differential sieving (mesh sizes 250, 106 and 75 μm (Fisons)). Slices of cortex in HBSS were transferred to the top sieve (250 μM) and gently mashed with the bulb end of a sterile pastette to break up the tissue and release the glomeruli, while repeatedly washing with HBSS. Glomeruli which collected on the lower two sieves (106 and 75 μm mesh size) were transferred to 2 universals and centrifuged for 5 minutes at 200 *g* at room temperature. The supernatant was removed and the glomeruli resuspended in 10 ml of prewarmed

sterile collagenase solution (type IV) (Sigma), 1 mg/ml in Waymouths medium. The glomeruli were incubated at 37°C for 20 minutes with occasional agitation, then centrifuged for 5 minutes at 200 *g*. The supernatant was removed and the glomeruli resuspended in 10 ml Waymouths medium containing 10% (v/v) foetal calf serum (FCS), and placed in two 80 cm^2 tissue culture flasks. Flasks were incubated at 37°C in 5% CO_2 and left undisturbed for 5 days, after which the media was changed. Primary cultures were established as outgrowths from glomeruli by day 10 and reached confluency by day 20.

2.1.2. Cell culture conditions

MC were routinely cultured in 80 cm^2 tissue culture flasks in Waymouths MB752/1 medium supplemented with antibiotics and 10% (v/v) FCS (referred to as complete medium). Cultures were maintained at 37°C in 5% CO_2 and 95% air. The medium was changed every 4 days.

To subculture, the medium was removed and the cells were washed 3x with 5 ml phosphate buffered saline (PBS) (w/o Ca^{2+} and Mg^{2+}). Cells were then incubated for approximately 5 minutes at 37°C in 3 ml of warmed 0.05% trypsin/0.02% EDTA (x1) (Gibco) until the cells had detached from the flask. Flasks were firmly tapped to encourage cell detachment. The action of the trypsin was inhibited by adding 10 ml of complete medium and the cells centrifuged at 200 *g* for 5 minutes. The cell pellet was resuspended in 1 ml of complete medium and the viable cells counted in a Neubauer haemocytometer (Weber Scientific International Ltd, Teddington, UK) after mixing 10 µl of resuspended cells with 40 µl of PBS and 10 µl of 0.4% trypan blue (Sigma). Dead cells stained blue, due to the uptake of trypan blue. The concentration of cells was calculated using the following equation;

cells / ml = (Number of cells in the centre 25 squares on each side of the haemocytometer x 6 x 10^4) ÷ 2

Cells were seeded into 80 cm^2 tissue culture flasks (12 ml/flask), 25 cm^2 flasks (4 ml/flask) or 24 multi-well plates (0.5 ml/well) at a density of 50,000 cells/ml. Plates and flasks reached confluency after approximately 3 and 5 days respectively.

For storage, cells between passages 0 and 3 were resuspended at $1x10^7$ cells/ml in Waymouths medium (no FCS) and mixed 1:1 with a cold solution of 80% FCS / 20% dimethylsulphoxide (DMSO) (final concentration = 40% FCS, 10% DMSO). The cell suspension was transferred to cryotubes (0.5 ml/tube) (Nunc) and gradually cooled in the vapour phase of liquid nitrogen over 6 hours. Cryotubes were then stored in liquid nitrogen tanks. When required, the cells were rapidly defrosted at 37°C in a water bath, washed in Waymouths medium and resuspended in complete medium. Cells from 1 cryotube were then plated into an 80 cm^2 flask.

2.1.3. Characterization of cells

MC identity was confirmed by the display of the characteristic hillock structures in culture (Sterzel *et al.* 1986) and by the use of a series of cell markers. For cell staining, MC were plated into sterile flat-bottomed 96 well plates or 8 well lab-tek chamber slides (Nunc) at a density of 25,000 cells/ml (100 and 300 µl/well respectively). Lab-tek slides were used if photographs were required. Cells were incubated for 48 hours prior to fixing. Sub-confluent cells were used, as the elongation that occurs when cells are confluent conceals clear staining of the intermediate filaments.

Cells to be fixed were washed 3x with PBS + 0.05% (w/v) BSA and air dried in a cell culture hood for 1 hour. 10% neutral buffered formalin solution (Sigma) was added and the cells incubated at 4°C for 1 hour. Cells were washed 3x with PBS + 0.05% BSA and left at 4°C prior to staining. Alternatively, cells to be stained for α-smooth-muscle specific actin were washed in PBS + 0.05% BSA and immediately fixed by immersing the cells for 10 minutes in cold methanol at -20°C.

Primary antibodies or phalloidin were appropriately diluted in PBS + 0.05% BSA and incubated with the cells for 1 hour (50 and 150 µl volumes were used in the 96-well plates and lab-tek slides respectively). Cells were washed 3x and the unlabelled primary antibodies detected by incubating for 1 hour with a 1:20 dilution of fluorescein

isothiocynate (FITC)-conjugated rabbit anti-mouse IgG (F313, Dako Ltd, High Wycombe, UK). Cells were washed 3x, and the lab-tek slides were mounted with a coverslip in glycergel mounting medium (Dako), which was left overnight to dry. Cells were viewed under ultraviolet light using a Nikon fluorescence microscope.

MC cultures stained positive for F-actin (1:100 dilution of phalloidin-FITC) (Sigma), α-smooth muscle specific actin (1:200 dilution of FITC-conjugated IgG2a monoclonal) (Sigma) (Fig. 10) and for the intermediate filament vimentin (1:25 dilution of IgG1k monoclonal) (M0725, Dako). This latter stain denotes cells of mesenchymal origin. Cultures at passage 3 to 4 were negative for cytokeratin (1:40 dilution of IgG2a monoclonal K8.13, Sigma) and von Willibrand factor antigen (1:25 dilution of IgG1k monoclonal, M0616, Dako) excluding contamination with epithelial and endothelial cells respectively. Cultures at passage 2 contained some epithelial cell contamination (<20%). Phalloidin-FITC was routinely used to assess the purity of cultures, as this reagent stains all the possible contaminating cells (fibroblasts, macrophages, endothelial and epithelial cells). MC cultures were first used in experiments at passage 3 or 4, when cultures were >95% pure. Cultures were negative when incubated with the same dilutions of mouse IgG1k control serum (X093, Dako) instead of the primary antibodies. The control serum was directed against Aspergillus niger glucose oxidase, which is not present in mammalian tissue. Specificity of the secondary FITC-conjugated antibody was demonstrated by omitting addition of the primary antibody on some wells. Furthermore, primary cultures of human synovial fibroblasts were used as a negative control for α-smooth muscle actin staining, while the epithelial cell line HT-29 was used as a positive control for cytokeratin staining. These latter 2 cell types were kindly supplied by Dr N. Jordan and Dr G. Kolios (Dept. of Pharmacology, University of Bath).

2.1.4. Mycoplasma assay

MC cultures were tested for contamination with the 4 common mycoplasma species (M. arginini, M. hyorhinis, A. laidlawii, M. orale) using an enzyme immunoassay kit (Boehringer Mannheim). The manufacturers protocol was followed for the detection of mycoplasma in culture supernatants, and all the buffers and reagents used were supplied with the kit.

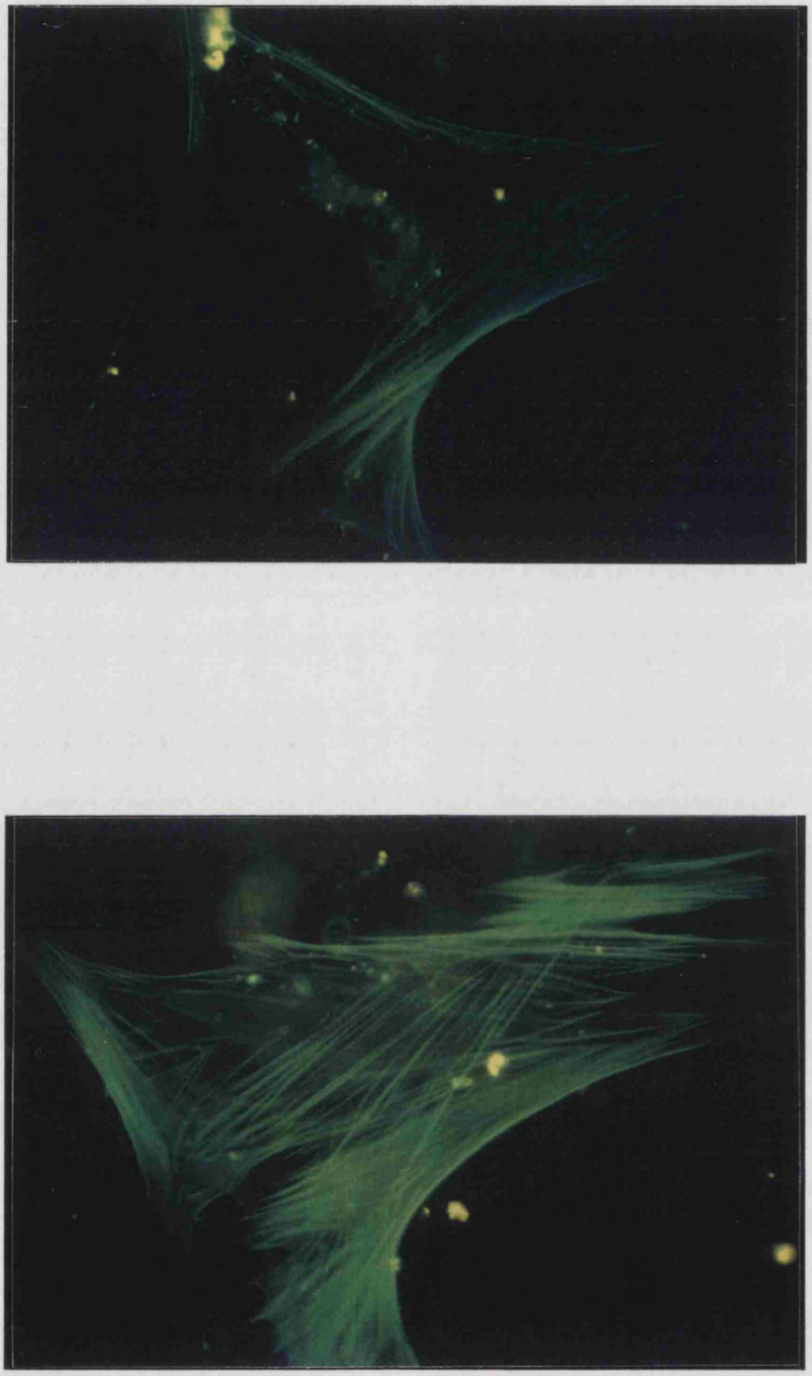


Figure 10. Human MC stained for smooth muscle specific actin. Sub-confluent MC grown in 8 well lab-tek chamber slides were methanol fixed prior to staining with a 1:200 dilution of FITC-conjugated anti-α-smooth muscle specific actin monoclonal antibody. Cells were photographed under a magnification of x400.

96 well microtitre plates (Nunc Immuno maxisorb plates) were coated with 125 µl of four different antibody solutions directed against the four mycoplasma species. Plates were incubated for 2 hours at 37°C. Antibody solutions were removed and non-specific binding sites blocked with 125 µl of blocking solution for 30 minutes at 37°C. Plates were washed 3x and incubated with either 100 µl of MC culture supernatant, positive control solutions or media alone, overnight at 4°C. Plates were washed 3x and incubated with 100 µl of the four corresponding biotin labelled detecting antibodies for 2 hours at 37°C. Plates were washed 3x and incubated with 100 µl of streptavidin-alkaline phosphatase for 1 hour at 37°C. Washed plates were incubated with 100 µl of substrate solution for 1 hour at room temperature. Results were evaluated visually and confirmed by reading optical densities at 405 nm.

Of 6 cultures tested during this study, 4 cultures were negative for all of the mycoplasma species screened for, while 1 culture was positive for M. arginini and another culture was faintly positive for M. arginini and M. hyorhinis. These later two cultures, which were both approximately 2 years old were discarded.

2.2. EXPERIMENTAL PROTOCOL

MC, between passages 3 to 7 were grown to 80% confluency in 24 multi-well tissue culture plates (~$5x10^4$ cells/well), 25 cm^2 flasks (~ $8x10^5$ cells/flask) or 80 cm^2 flasks (~ $2.5x10^6$ cells/flask). Twenty-four hours prior to the start of the experiment the cells were washed and cultured in serum-free Waymouths medium. MC were then treated with fresh serum-free medium and challenged with either drugs, cytokines or vehicle controls for the times and doses specified in the results section. Supernatants were collected, centrifuged to remove cellular debris and stored at -70°C until assayed for extracellular IL-6, IL-8, RANTES, PGE_2 or nitrite. Total cellular RNA, cell membranes or cellular proteins were extracted from cells as described in sections 2.8.1, 2.9.1 and 2.10.2 respectively. Cell viability was routinely assessed at the end of the experiment by phase microscopy and by trypan blue exclusion.

2.3. PROTEIN ASSAY

Protein was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), which is based on the coomassie brilliant blue dye-binding method of Bradford (Bradford, 1976).

The protein content of samples was estimated by comparison with a standard curve of bovine gamma globulin (Bio-Rad standard 1) ranging from 0-25 μg/ml in PBS. 200 μl/well of standard and appropriately diluted sample (e.g. MC membranes, IL-6 antibody) were added to a flat-bottomed 96-well microtitre plate in duplicate. 50 μl of Bio-Rad dye-binding reagent was added to each well and the samples were mixed using a multi-channel pipette. After 20 minutes the absorbance was measured at 595 nm.

2.4. AFFINITY PURIFICATION OF ANTI-IL-6 ANTIBODY

Goat anti-human IL-6 antiserum (Bleeds G150 B3a and G150 BM) and human recombinant IL-6 (hrIL-6) were a gift from Dr Steven Poole (National Institute of Biological Standards and Controls (NIBSC), South Mimms, UK). The antiserum was affinity purified and biotinylated as previously described (Rafferty *et al.* 1991; Taktak *et al.* 1991) for use in an IL-6 ELISA.

2.4.1. Preparation of column for affinity purification

Human recombinant IL-6 was immobilised on affigel 15, a matrix of cross-linked agarose containing N-hydroxysuccinimide functional groups, which bind amino ($-NH_2$) groups in proteins spontaneously via a covalent bond. Affigel 15 solution (Bio-Rad) was transferred to a 15 ml sterile conical tube (Falcon, Becton Dickinson, Oxford, UK) to give a 1 ml volume of settled gel. The affigel was centrifuged at 180 *g* for 5 minutes and the supernatant removed and discarded. The gel was washed in 3 ml of cold double-distilled sterile water and centrifuged. The supernatant was removed and 1 mg of hrIL-6 in 4 ml of cold PBS was added. For optimum coupling, this first part of the procedure was performed within 20 minutes. The gel slurry was then agitated on a tube

rotator for 4 hours at 4°C. After 4 hours, the affigel was centrifuged and the supernatant removed and kept to be assayed for unbound hrIL-6. The affigel was washed twice with 3 ml double-distilled water and both supernatants were kept to assay for unbound IL-6. The affigel was transferred to a column consisting of a 2 ml sterile syringe body with a cotton wool plug and a two-way valve. The column was washed with double-distilled water, followed by 20 ml 0.1 M glycine/HCl pH 2.5, the solution to be later used to elute the antibodies from the column. Finally, the column was washed with PBS and stored at 4°C in PBS + 0.1% sodium azide.

Unbound IL-6 in wash supernatants was calculated from the absorbance at 280 nm in a 1 cm light path. The pH of the supernatants were first lowered using HCl, as N-hydroxysuccinimide released during coupling adsorbs at neutral or basic pH.

$$\text{IL-6 (mg/ml) in supernatant} = \frac{\text{A280 nm x 10}}{13.6}$$

Based on the above equation (Harlow & Lane, 1988), only 18% of the hrIL-6 remained unbound.

2.4.2. Purification of IL-6 antibody

The IL-6 affigel column was washed with 50 ml PBS at room temperature, prior to the addition of 1 ml of IL-6 antiserum. The antiserum was allowed to penetrate completely into the gel, before the flow was stopped and PBS added to the top of the gel to prevent it drying out. The column was covered with nescofilm and incubated at room temperature for 4 hours. The column was washed with 50 ml PBS to remove non-specific antibodies. Specific anti-IL-6 antibody was eluted with 10 ml 0.1 M glycine/HCl, pH 2.5 and 1 ml fractions were collected in tubes containing 200 µl 1 M TRIS, pH 8.8 to neutralise the pH of the sample. The column was then washed with a further 10 ml glycine/HCl followed by PBS. The column was stored in PBS + 0.1% sodium azide at 4°C.

The protein concentration of each fraction was determined by reading the absorbance at 280 nm. The first 4 - 6 fractions contained the eluted IL-6 antibody and were therefore pooled. The antibody was dialysed overnight at 4°C against either 3 L of PBS, pH 7.4 (for ELISA coating antibody) or 100 mM HEPES, pH 8.5 (for detecting antibody). The protein concentration of the antibody solutions was measured using a Bio-Rad protein assay (see section 2.3). 1 and 1.7 mg of antibody was obtained per ml of serum from two separate bleeds. Samples in PBS were then concentrated to 1 mg/ml in an Amicon Ultrafiltration cell, under nitrogen gas, using a YM 30 membrane (Diaflo ultrafiltration membranes, Amicon Corporation, Danvers, USA) and stored at -20°C. Antibody in 100 mM HEPES was concentrated to 2 mg/ml ready for biotinylation.

2.4.3. Biotinylation of antibodies

Antibodies (anti-IL-6 and anti-RANTES) were labelled using a succinimide ester of biotin, which covalently couples to the protein via free amino groups. Biotinylated antibodies can then be detected using peroxidase labelled streptavidin, which has a very high affinity for biotin.

The protein to be biotinylated was dissolved in 100 mM HEPES buffer, pH 8.5 to a concentration of 2 - 10 mg/ml (e.g. to a 1 ml volume of a 2 mg/ml solution). Immediately prior to use, N-hydroxysuccinimidobiotin (Sigma) was dissolved in warmed N,N-dimethylformamide (DMF) (Aldrich) to an appropriate concentration (e.g. 10 mg/ml), so that an amount of biotin equivalent to 1/10 of the weight of protein (e.g. 0.2 mg biotin) was added in a 20 µl volume to the antibody preparation. The preparation was incubated at room temperature for 2 hours with occasional agitation. The reaction was stopped by adding 5 µl ethanolamine (Sigma) and the biotinylated antibody was dialysed against 3 changes of 2 L of PBS, pH 7.4 to remove unreacted biotin. The antibody was stored at -20°C.

2.5. ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

Recipes for the buffers used in the following ELISA protocols are given in appendix III. Standard curves were run on every plate, and a control standard of recombinant IL-6, IL-8 or RANTES diluted in wash buffer + 2% FCS (stored frozen, in aliquots at -20°C) was included on each plate to monitor both interplate and interassay variability.

2.5.1. IL-6 ELISA

Antigenic IL-6 peptide in culture supernatants was quantitated using a double-ligand ELISA method, as previously described (Taktak *et al.* 1991). Optimum concentrations of antibody for coating and detecting were determined for each batch of antibody purified by performing a checkerboard ELISA to compare different coating and detecting antibody concentrations (in the range of 0.5 - 1.5 μg/ml). The concentrations which produced a steeper standard curve were used. IL-6 in cell supernatants was estimated by comparison with a standard curve of the 'international standard for IL-6' (code 89/548, NIBSC) (Gaines Das & Poole, 1993).

Flat-bottomed 96-well microtitre plates (Nunc Immuno maxisorb plates) were coated with 100 μl/well of affinity purified anti-IL6 antibody in PBS coating buffer. Plates were covered and incubated overnight at 4°C. Plates were washed 3x with wash buffer, followed by the addition in duplicate of either 100 μl of IL-6 standard (range 0.008 to 2 ng/ml) or culture supernatant appropriately diluted in wash buffer + 2% (v/v) FCS, and incubated for 2 hours at room temperature. Plates were washed 3x and 100 μl biotinylated anti-IL-6 antibody diluted in wash buffer + 2% FCS was added to all wells and incubated for 1 hour at room temperature. Plates were washed 3x and incubated with 100 μl streptavidin-peroxidase (Sigma) (0.5 μg/ml) diluted in wash buffer + 2% FCS for 15 minutes. Following 3 washes, 100 μl of 0.2 mg/ml *o*-phenylenediamine dihydrochloride (OPD) dissolved in warm 0.1 M citric acid-phosphate buffer containing 0.4 μl/ml 30% (v/v) H_2O_2 was added to all wells. The substrate solution was prepared immediately prior to use. After 15 minutes incubation in the dark at room temperature, colour development was stopped with 150 μl 1 M H_2SO_4 and optical density (OD) measured at 492 nm. The assay was linear between 0.03 - 2 ng/ml (Fig. 11).

2.5.2. IL-8 ELISA

Antigenic IL-8 in culture supernatants was quantitated using a double-ligand ELISA method as previously described (Ceska *et al.* 1989). Mouse monoclonal anti-human IL-8 antibody, goat polyclonal anti-human IL-8 antibody conjugated to alkaline phosphatase and human recombinant IL-8 standard were provided by Dr I. Lindley of Sandoz Forschungsinstitut (Vienna, Austria). Antibodies were stored at 4°C.

96 well microtitre plates (Nunc immuno maxisorb plates) were coated with 100 µl/well of monoclonal anti-IL-8 antibody (5 µg/ml) in carbonate coating buffer. Plates were covered and incubated overnight at 4°C. Plates were washed 3x with wash buffer, followed by the addition in duplicate of 100 µl IL-8 standard (0.025 to 2 ng/ml) or culture supernatant appropriately diluted in wash buffer + 2% (v/v) FCS, and incubated for 2 hours at 37°C. Plates were washed 3x and 50 µl alkaline phosphatase-conjugated goat anti-IL-8 antibody (5 µg/ml) in wash buffer + 2% FCS was added for 2 hours at 37°C. Following 3 washes, plates were incubated with 100 µl of 1 mg/ml *p*-nitrophenyl phosphate in warmed diethanolamine buffer at room temperature, until the top standard read ~1.5 OD. The reaction was terminated with 50 µl/well 3 M NaOH, and the OD measured at 405 nm. The assay was linear between 0.05 - 2 ng/ml (Fig. 12).

2.5.3. RANTES ELISA

Antigenic RANTES in culture supernatants was quantitated using a double-ligand ELISA method. Mouse monoclonal anti-human RANTES antibody and goat polyclonal anti-human RANTES antibody were purchased from R&D systems (Abingdon, UK). The polyclonal anti-RANTES antibody was biotinylated according to the method described in 2.4.3, and both monoclonal and polyclonal antibodies were stored in aliquots at -20°C. Human recombinant RANTES peptide was from PeproTech INC, Rocky Hill, NJ.

96 well microtitre plates (Nunc immuno maxisorb plates) were coated with 50 µl/well of monoclonal anti-RANTES antibody (1 µg/ml) in carbonate coating buffer, pH 9.6. The plates were covered and incubated overnight at 4°C. Plates were washed 3x with

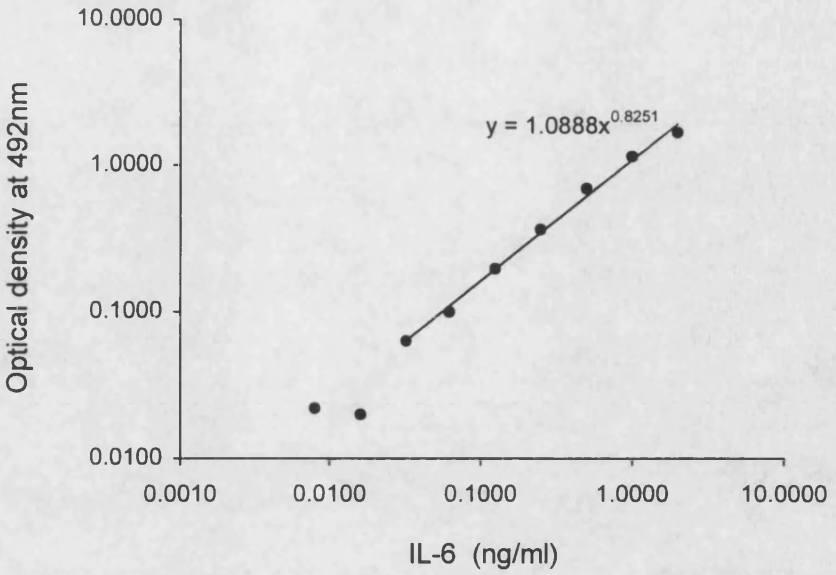


Figure 11. Standard curve for the IL-6 ELISA. OD values are the means of duplicates after subtracting the average OD of blank wells. The graph is plotted on a log-log scale. A 0.5 ng/ml IL-6 control standard had a value of 0.51 ± 0.01 ng/ml (mean ± SEM) after 9 determinations on different days.

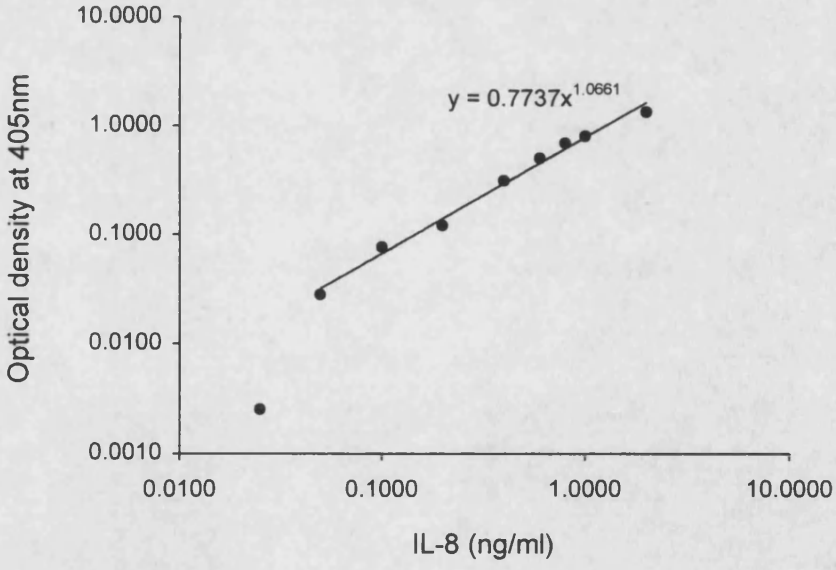


Figure 12. Standard curve for the IL-8 ELISA. OD values are the means of duplicates after subtracting the average OD of blank wells. The graph is plotted on a log-log scale. A 0.8 ng/ml IL-8 control standard had a value of 0.70 ± 0.01 ng/ml (mean ± SEM) after 11 determinations on different days.

wash buffer, followed by the addition in duplicate of 50 µl of RANTES standard (0.06 to 2 ng/ml) or culture supernatant diluted in wash buffer + 2% FCS, and incubated for 2 hours at 37°C. Plates were washed 3x and 50 µl biotinylated polyclonal anti-RANTES antibody (1 µg/ml) in wash buffer + 2% FCS added for 2 hours at 37°C. Plates were washed 3x and incubated with 50 µl streptavidin-peroxidase (0.5 µg/ml) in wash buffer + 2% FCS for 30 minutes. Following 3 washes, 100 µl of 0.2 mg/ml OPD dissolved in warm 0.1 M citric acid-phosphate buffer containing 0.4 µl/ml 30% (v/v) H_2O_2 was added. After a 20 minute incubation in the dark, at room temperature, the reaction was quenched with 150 µl 1 M H_2SO_4 and the OD at 492 nm was determined. The assay was linear between 0.06 - 2 ng/ml (Fig. 13).

2.6. RADIOIMMUNOASSAY FOR MEASUREMENT OF PROSTAGLANDIN E_2

PGE_2 levels in cells supernatants were quantitated using a commercial PGE_2 [^{125}I] radioimmunoassay kit (NEN research products, Boston, USA). The basic principle of the assay is competitive-binding. The protocol recommended by the manufacturers was followed and the detection range of the assay was 2.5 - 250 pg/ml. Briefly, either samples or PGE_2 standards were incubated overnight at 4°C with a fixed, limiting amount of anti-PGE_2 rabbit serum and a fixed amount of iodinated analogue of PGE_2 as a tracer. Antibody-antigen complexes were separated from free antigen by precipitation with polyethylene glycol, and the amount of PGE_2 [^{125}I] tracer bound was determined by counting each sample for 1 minute in a gamma counter. The concentration of PGE_2 in the samples was determined from a standard curve.

2.7. NITRITE ASSAYS

Nitric oxide (NO) production by MC was assessed by measuring nitrite (NO_2^-) and nitrate (NO_3^-) levels in culture supernatants. These are the stable end products of NO formation in oxygenated solution.

2.7.1 Griess Reaction

Nitrite levels of culture supernatants were initially quantitated using the Griess reaction (Green *et al.* 1982). The assay is based on the reaction of nitrite with Griess reagent which forms a purple azo dye, that absorbs at 546 nm.

Griess reagent was made from stock solutions of 0.1% (w/v) naphthylenediamine dihydrochloride (Sigma) in milli-Q water and 1% (w/v) sulphanilamide (Sigma) in 5% phosphoric acid. The stock solutions were stored in the dark at 4°C for upto 2 months. On the day required, the two solutions were mixed 1:1 and warmed to room temperature. A standard curve ranging from 0.78 - 100 µM was freshly prepared from sodium nitrite (Sigma) diluted in Waymouths medium. 100 µl/well of nitrite standards and samples were added in duplicate to a 96 well microtitre plate and mixed with 100 µl of Griess reagent. After 10 minutes the OD at 540 nm was determined. The detection limit of the assay was 0.8 µM.

Waymouths medium, which contains phenol red did not interfere with the assay (Fig. 14) and this medium contains no added nitrite or nitrate.

2.7.2. Fluorimetric assay

The fluorimetric nitrite assay is based upon the reaction of 2,3-Diaminonaphthalene (DAN) (Lancaster, Morecambe, UK) with nitrite under acidic conditions to form the fluorescent product 1-(H)-naphthotriazole. The assay was modified for use on a Photon Technology International (PTI) spectrofluorimeter from the method of Misko *et al* (1993), which employed a 96 well plate format for a fluorescent plate reader. In contrast to the plate reader, the optimum excitation and emission wavelengths for the assay could be set on the fluorimeter, thus improving sensitivity. Fluorescent excitation and emission spectra for 1-(H)-naphthotriazole were obtained as previously described (Misko *et al.* 1993), to determine the optimum wavelengths for the assay using our system. Nitrite standards made up in milli-Q water were reacted with DAN reagent as described below and scanned. The emission wavelength was set at 410 nm to obtain an excitation spectrum (Fig. 15) and a 365 nm excitation wavelength was used to obtain an

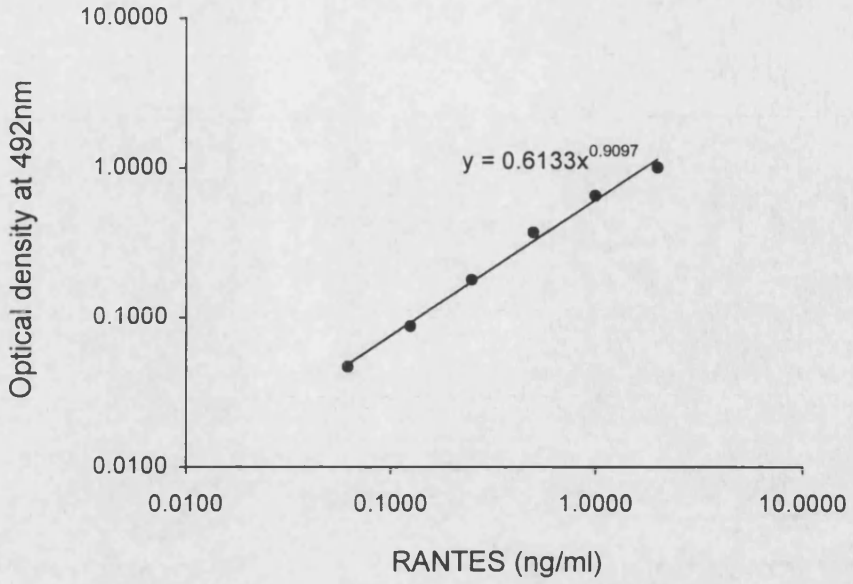


Figure 13. Standard curve for the RANTES ELISA. OD values are the means of duplicates after subtracting the average OD of blank wells. The graph is plotted on a log-log scale. A 1.5 ng/ml RANTES control standard had a value of 1.4 ± 0.19 ng/ml (mean ± SEM) after 6 determinations on different days.

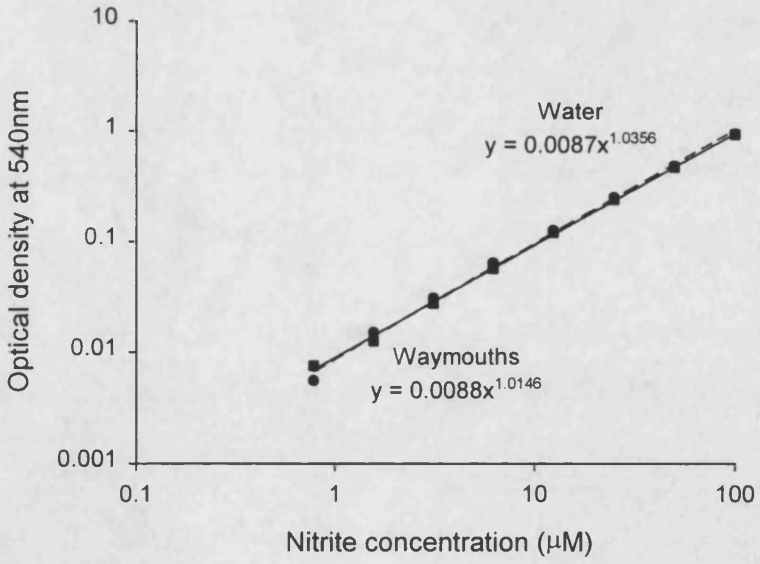


Figure 14. Standard curve for the Griess reaction in water or Waymouths medium. Sodium nitrite standards were either diluted in MQ-water (•, ___) or Waymouths medium (■, -----). OD values are the means of duplicates after subtracting the average OD of blank wells. The graph is plotted on a log-log scale.

emission spectrum (Fig. 16). The scan speed was 240 nm/min with excitation and emission slits set at 10 nm. An excitation wavelength of 365 nm and emission wavelength of 405 nm were found to be optimum, which was consistent with previous findings (Misko *et al.* 1993).

Milli-Q water and sterile glassware were used to prepare all reagents for the assay to keep background levels of nitrite low. A standard curve of sodium nitrite in Waymouths medium, ranging from 100 nM to 2 μM was prepared. 2 ml of standard or culture supernatant was mixed in a bijoux with 200 μl of freshly prepared DAN reagent (0.05 mg/ml DAN in 0.62 M HCl) and incubated at room temperature in the dark. After 10 minutes the reaction was stopped by the addition of 100 μl 2.8N NaOH. Fluorescent intensity of 2 ml volumes of standards and samples was measured on the fluorimeter using an excitation wavelength of 365 nm and an emission wavelength of 405 nm.

Waymouths medium, which contains phenol red caused some interference with the assay, reducing fluorescence levels compared to water (Fig. 17).

2.7.3. Chemiluminescence assay

Nitrite levels in cell supernatants were also quantitated by chemiluminescence (Garside, 1982; Menon *et al.* 1991), using a Sievers NOA 27OB nitric oxide analyser (Boulder, Colorado, USA). The assay was kindly performed by Dr Mark Wilkes (Queen Elizabeth hospital, Birmingham), and detects nitrite levels down to the pM range. Chemiluminescent measurement is based on the gas phase reaction between NO and ozone, which generates light that is detected by a sensitive photomultiplier tube. This technique measures NO directly, therefore nitrite levels in culture supernatants were first converted back to NO during the assay by the addition of 1% sodium iodide solution in glacial acetic acid. An inert gas (nitrogen) is then bubbled through the supernatant to push the NO into the gas phase, which is achieved due to the low solubility of NO in aqueous solution. Five separate measurements were performed for each sample, using 100 μl supernatant per measurement. The assay was calibrated using standards of sodium nitrite.

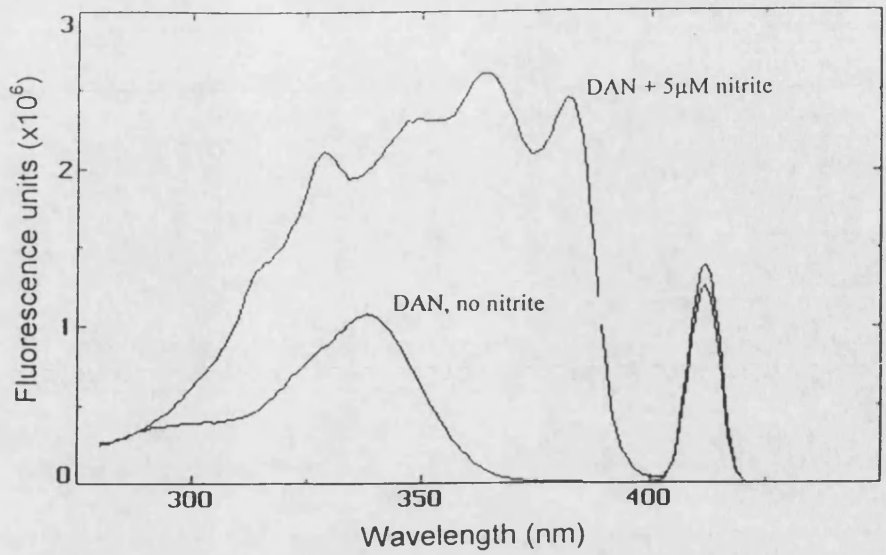


Figure 15. Excitation spectrum of the fluorescent product, 1-(H)-naphthotriazole. A 5 μM nitrite standard was reacted with DAN reagent and scanned using an emission wavelength of 410 nm. The scan speed was 240 nm/min with excitation and emission slits set at 10 nm.

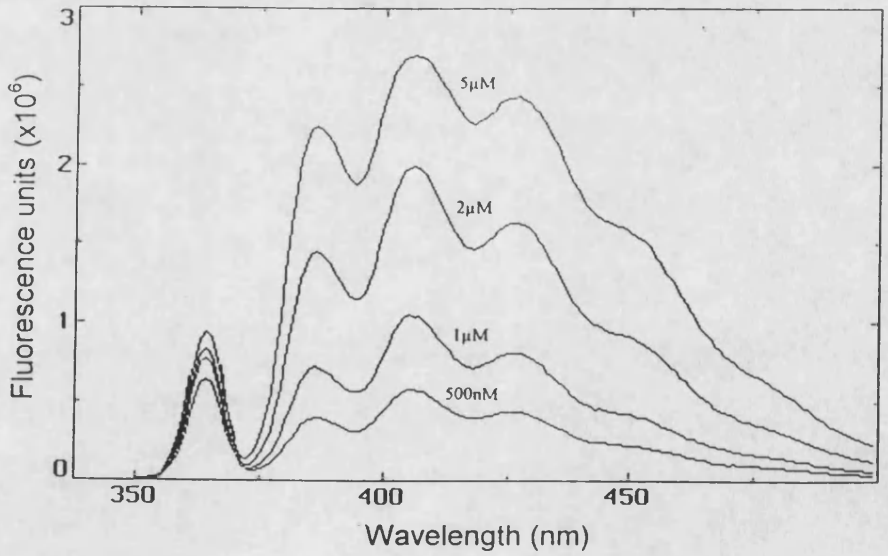
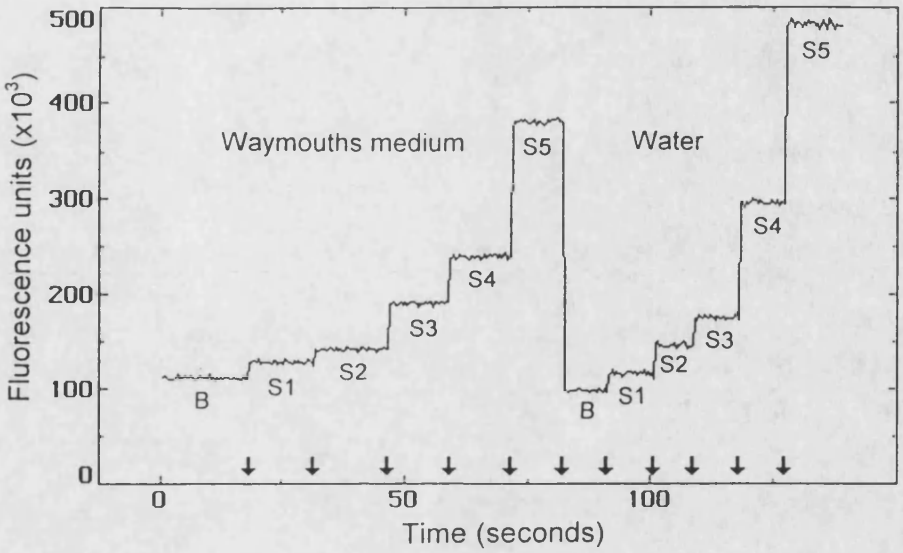


Figure 16. Emission spectrum of 1-(H)-naphthotriazole. Nitrite standards (0.5 - 5 μM) were reacted with DAN reagent and scanned using an excitation wavelength of 365 nm. The scan speed was 240 nm/min with excitation and emission slits set at 10 nm.

a)



b)

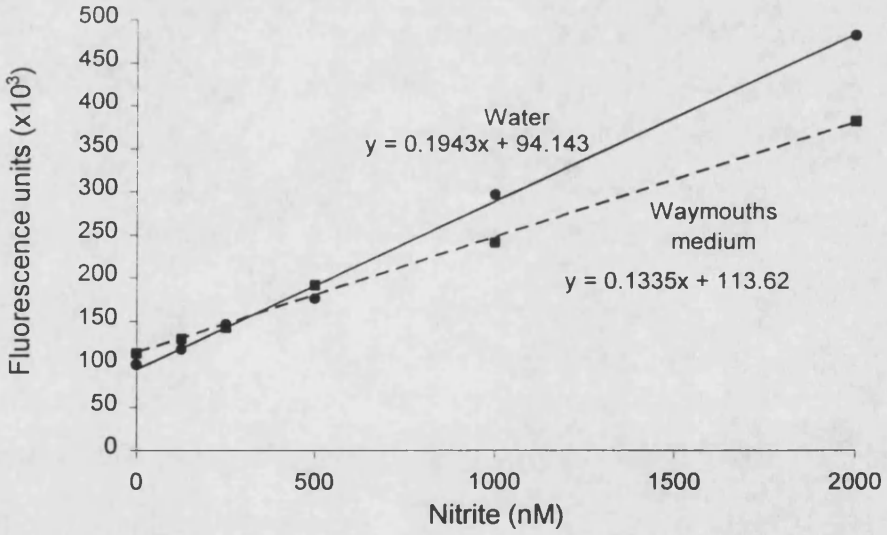


Figure 17. Standard curve for the fluorimetric nitrite assay in water or Waymouths medium. Sodium nitrite standards (B=blank, S1=100 nM, S2=250 nM, S3=500 nM, S4=100 nM, S5=2000 nM) were either diluted in MQ-water (•, —) or Waymouths medium (■, ----). Fluorescence values were read off the fluorimeter trace (a) and plotted against nitrite concentrations using a linear scale (b).

2.7.4. Enzymatic conversion of nitrate to nitrite

Nitrate levels in culture supernatants are not detected by the Griess reaction or fluorimetric assay. Nitrate must therefore be reduced to nitrite for measurement. Nitrate was reduced to nitrite using the enzyme nitrate reductase from *Aspergillus niger*, as previously described (Misko *et al.* 1993; Hevel & Marletta, 1994).

Reagents:

ß-Nicotinamide adenine dinucleotide phosphate - reduced form (ß-NADPH; Sigma) was diluted to 0.6 mM in 0.15 M potassium phosphate buffer, pH 7.5 and stored in aliquots at -20°C for upto 2 weeks.

Nitrate reductase (NADPH-dependent) (Sigma) was diluted to 4.2 U/ml in 0.15 M potassium phosphate buffer and stored in aliquots at -20°C.

Milli-Q water and sterile glassware were used to prepare all buffers to reduce background nitrate levels.

Culture supernatants were incubated with 280 mU/ml nitrate reductase and 40 µM NADPH for 30 minutes at room temperature. Samples to be measured by the Griess reaction were incubated in a final volume of 100 µl, in duplicate, in a 96 well plate. The reaction was terminated after 30 minutes by the addition of 100 µl of Griess reagent and nitrite levels were estimated against a standard curve of sodium nitrite as described in section 2.10.1. Samples to be measured by the fluorimetric assay were incubated in a final volume of 300 µl and the reaction was terminated after 30 minutes by the addition of 300 µl of milli-Q water. The samples were then reacted as described in 2.10.2, with appropriately adjusted volumes of DAN reagent and 2.8N NaOH (60 µl and 30 µl respectively). The fluorescent intensity of 600 µl of converted samples and standards were measured in the fluorimeter using a small quartz cuvette (650 µl capacity). The % conversion achieved each time was calculated by converting standards of sodium nitrate (Sigma) in Waymouths medium in parallel with samples.

2.8. NORTHERN ANALYSIS

Total cellular RNA was isolated from MC using a modification of the methods of Chirgwin *et al* (1979) and Strieter *et al* (1989). Buffers and solutions used for northern analysis are detailed in appendix IV, and all solvents were 'analytical reagent' grade.

2.8.1. RNA isolation

MC, in 25 cm^2 tissue culture flasks, were scraped into 3 ml nucleic acid extraction buffer and frozen at -70°C in 15 ml sterile polypropylene conical tubes (Falcon) for at least 1 hour. RNA was separated from DNA and protein using phenol-chloroform and isoamyl alcohol-chloroform. The tubes were kept on ice during the following steps. The cell mixture was homogenised by pipetting up and down 25x using a sterile pastette. 1.5 ml of phenol extraction buffer and 1.5 ml of a 1:1 solution of water-saturated phenol (Rathburn chemicals Ltd, Walkerburn, UK):chloroform was added and each tube vortexed vigorously for 2 minutes. Tubes were centrifuged at 3000 *g* for 10 minutes at 4°C. Using a sterile glass pipette, the upper aqueous layer was transferred to a fresh 15 ml tube and 1.5 ml phenol : chloroform added. The tubes were vortexed and centrifuged as before. The upper aqueous layer was transferred to a fresh 15 ml tube and 2.5 ml of a 1:25 solution of isoamyl alcohol : chloroform added. The tubes were vortexed and centrifuged at 3000 *g* for 7 minutes. The upper aqueous layer was transferred to a fresh 15 ml tube and mixed with 300 µl 3 M sodium acetate and 3 ml propan-2-ol. Tubes were kept at -70°C for at least 1 hour to allow RNA to precipitate. The contents were then transferred to a 16 x 76 mm polyallomer ultra centrifuge tube (Beckman Instruments Ltd, Buckinghamshire, UK) which had been DEPC-treated and autoclaved. The tubes were centrifuged at 30,000 *g* for 75 minutes at 4°C. The supernatant was removed and the RNA pellet transferred to an eppendorf tube in 1.5 ml 75% ethanol solution. The RNA pellets were frozen at -70°C for at least 1 hour.

2.8.2. Sample preparation

RNA samples were centrifuged at 12,000 *g* for 15 minutes at 4°C. The supernatant was removed and 1.5 ml 75% ethanol added to the RNA pellet. The samples were centrifuged and the wash step repeated. The supernatant was removed and the pellets

air dried in a fume cupboard for 1 hour. RNA pellets were resuspended in 25 µl diethyl pyrocarbonate (DEPC) treated water and kept on ice. RNA was quantitated by measuring the absorbance of 2 µl RNA in 1 ml 0.1 M NaOH at 260 nm. The amount of RNA present in the sample (in µg) was calculated by;

$OD_{260\,nm}$ x dilution factor (500) x 40 x volume of remaining RNA solution in ml (0.023)

ODs were also read at 280 nm and 230 nm to assess the purity of the RNA. A value of less than 2 for the OD_{260} : OD_{280} ratio indicates protein contamination. A low (<2) OD_{260} : OD_{230} ratio indicated guanidine contamination.

33 µl of RNA sample buffer was added to 10 µg RNA and the samples were vortex mixed and heated for 15-30 minutes at 80°C. The samples were cooled on ice and 2.5 µl of bromophenol blue solution added. Samples were mixed and briefly centrifuged (5 seconds) prior to loading on to agarose gels.

2.8.3. Gel preparation and transblotting

A 1% agarose gel was prepared by dissolving 3 g agarose (Boehringer Mannheim) in 230 ml DEPC-treated water. The solution was heated until the agarose dissolved. 15 ml 20x 3-[N-morpholino]propane-sulphonic acid (MOPS) buffer and 54 ml formaldehyde were added and the gel solution allowed to cool to ~60°C before pouring. The gel was set with two 15 lane combs using tanks purchased from Hoefer Scientific Instruments (Newcastle, UK). After 40 minutes, the gel was transferred to a submarine tank (Hoefer), which was surrounded with ice, and covered with 1 L of cold 1x MOPS running buffer. 10 µg RNA per lane was loaded and the gel run at a constant current of 100 mA, until the bromophenol blue band had migrated 1.5 inches (~2 hours). The gel was observed under UV light and the ethidium-bromide stained 18S and 28S ribosomal RNA bands used to assess equal loading. The gel was photographed using a polaroid CU5 88-46 land camera (Genetic research instrumentation Ltd) and type 55 polaroid film (Sigma), before being agitated in DEPC-water for 30 - 60 minutes to remove the formaldehyde, prior to transblotting.

The blotting tank consisted of a glass plate suspended in a sandwich box, which was half filled with 20x SSC buffer. A wide strip of filter paper placed over the glass plate and reaching down into the buffer solution at each end acted as a wick. The gel was placed upside down on the filter paper and covered with a piece of positively charged nylon membrane (Boehringer Mannheim), which had been briefly soaked in 20x SSC. All air bubbles were removed before covering the membrane with 3 pieces of similar size filter paper and a stack of paper towels. A glass plate and 500 g weight were placed on top, and left overnight to allow the RNA to transfer by capillary action. The RNA was fixed onto the nylon membrane by baking at 120°C for 20 minutes. The membrane was sealed in a plastic bag and stored at room temperature prior to hybridisation.

2.8.4. Hybridisation and detection using the non-radioactive DIG-labelling system

The DIG detection system is based on the labelling of nucleic acid probes with a steroid hapten, digoxigenin. The DIG-labelled probes are hybridised to membrane-bound RNA. Specific hybridisation is immuno-detected with an alkaline phosphatase conjugated anti-digoxigenin antibody and visualised with the chemiluminescent substrate, CSPD (see Abbreviations, p.viii), using X-ray film.

All reagents used for the detection of DIG-labelled probes were purchased from Boehringer Mannheim. DIG-labelled cocktails of oligonucleotide probes to IL-8, MCP-1, RANTES, IL-6, iNOS and ß-actin were purchased from R&D systems. Hybridisation of membranes and detection of bound probes was performed essentially as described in the Boehringer Mannheim protocols. Hybridisation temperatures were optimised for each probe. IL-8, MCP-1, RANTES, IL-6 and ß-actin probes were all hybridised at 42°C, while the iNOS probe required a temperature of 60°C. All probes were used at a final concentration of 10 ng/ml.

The volumes specified are for a 100 cm^2 membrane and pre-hybridisation, hybridisation and stringency wash steps were all performed at the appropriate hybridisation temperature. The membrane was pre-hybridised by incubating with 20 ml hybridisation solution for 1 hour in a sealed plastic bag. The solution was discarded and 2.5 ml of probe diluted to 10 ng/ml in hybridisation solution added. All air bubbles were

removed and the bag resealed and incubated overnight. The membrane was transferred to a small sandwich box and stringency washes performed. The membrane was washed twice for 5 minutes in 2x SSC + 0.1% SDS solution followed by 2x 5 minutes in 0.1x SSC + 0.1% SDS solution. The following steps were then performed at room temperature on a shaking waterbath. Membranes were washed for 5 minutes in wash buffer, prior to blocking for 30 minutes with 100 ml buffer 2. Membranes were incubated for 30 minutes with 20 ml alkaline phosphatase conjugated anti-DIG antibody diluted 1:10,000 in buffer 2, washed 2x15 minutes in wash buffer and equilibrated for 2-5 minutes in buffer 3. The membrane was drained and incubated for 5 minutes between 2 plastic sheets with 1 ml CSPD substrate diluted 1:100 in buffer 3. The membrane was drained, sealed in a plastic bag and incubated at 37°C for 15 - 30 minutes. The membrane was then exposed to Kodak omat AR5 X-ray film (Sigma) for 1 to 2 hours at room temperature.

2.8.5. Stripping and reprobing membranes

In some experiments the probe was removed from northern blots which had been developed as described in 2.8.4, and the membranes were reprobed with the house keeping gene β-actin to determine equal loading and assess specificity of drug treatments.

Membranes were rinsed thoroughly in DEPC-water and incubated for 2 x 30 minutes in 50% N,N-dimethylformamide, 1% SDS, 50 mM TRIS-HCl, pH 8.0 at 68°C. Membranes were rinsed in DEPC-water, then in 2x SSC and prehybridized and hybridized as described above.

2.9. ADP-RIBOSYLATION EXPERIMENTS

2.9.1. Preparation of cell membranes

Cell membranes were extracted from MC grown in 80 cm^2 tissue culture flasks for use in the ADP-ribosylation experiments. MC used in the toxin time course experiments described in 2.9.2.1 were pretreated with either CT (100 ng/ml) or PT (1 μg/ml) for 0 - 6

hours prior to membrane extraction. 2 flasks per time point were used. After toxin treatment, cells were washed 2x with PBS and trypsinised off the flasks, as detailed in 2.1.2. The pellet of cells was washed 2x with PBS to remove traces of medium and FCS added to inhibit the action of trypsin.

Membranes were extracted from untreated MC for use in the agonist-dependent cholera toxin ribosylation experiment described in 2.9.2.2. Cells were washed 3x in PBS and scraped off the flasks into 6 ml PBS containing a cocktail of protease inhibitors (pepstatin A, aprotinin and leupeptin at 10 μg/ml, phenylmethylsulfonyl fluoride (PMSF) at 174 μg/ml (Sigma)). Cells were scraped from the flasks to ensure cell receptors remained intact. Cells were pelleted by centrifuging at 500 *g* for 10 minutes at 4°C.

Cell membranes were then extracted as previously described (O'Neill *et al.* 1990a). Cells were resuspended in 1 ml hypotonic buffer (10 mM Tris, 1 mM $MgCl_2$, pH 7.2) containing a cocktail of protease inhibitors (as above), and mixed with 4 volumes of sucrose buffer (0.25 M sucrose dissolved in hypotonic buffer). Membranes were homogenised by 50 strokes in a 'tight' dounce pestle and centrifuged at 500 *g* for 5 minutes at 4°C to remove nuclei and unbroken cells. A membrane enriched fraction was obtained by centrifuging the supernatant at 25,000 *g* for 30 minutes at 4°C. The resulting membrane pellets were resuspended in 1 ml PBS containing 1 mM PMSF. The protein content of samples was determined as described in section 2.3. Membranes were stored at -70°C for use in the toxin time course experiments, or overnight at 4°C for use in the agonist-dependent ribosylation experiments.

2.9.2. ADP-ribosylation assay

Stock solutions for ADP-ribosylation assay: All solutions were prepared fresh on the day in double-distilled water, except for 50 mM $MgCl_2$, which was stored.

100 mM thymidine (Sigma)
30 mM ATP (disodium salt, grade I) (Sigma)

10 mM GTP (sodium salt, type III) (Sigma)

10 μM β-NAD (Sigma)

1 M dithiothreitol (DTT) (Gibco)

50 mM $MgCl_2$

1 M Arginine-hydrochloride (Sigma)

2.9.2.1. *Cholera and pertussis toxin time course experiments*

Extracted membranes were exposed to either cholera toxin, cholera toxin B oligomer, pertussis toxin or pertussis toxin B oligomer in the presence of a [^{32}P]-NAD label (NEN research products) and the level of ribosylation determined as previously described (O'Neill *et al.* 1992). All toxins were pre-activated immediately prior to use by incubating with 10 mM dithiothreitol for 20 minutes at 30°C to release the S1 subunit, then kept on ice. Cell membranes were washed with 0.15 M potassium phosphate buffer, pH 7.5 and collected by centrifuging at 10,000 *g* for 5 minutes. 60 μg of membrane protein was incubated with 50 μg/ml of pre-activated CT or CT-B, or with 10 μg/ml pre-activated PT or 100 μg/ml pre-activated PT-B in 0.15 mM potassium phosphate, 10 mM thymidine, 0.5 mM ATP, 50 μM GTP, 10 μM [^{32}P]-NAD^+ (50 - 100 μCi/ml) in a final volume of 100 μl. After incubating for 15 minutes at 30°C, the reaction was stopped by the addition of 1 ml of cold 0.15 M potassium phosphate buffer and membranes were collected by centrifuging at 10,000 *g* for 5 minutes at 4°C. Membranes were resuspended in 30 μl 1x electrophoresis sample buffer and proteins were separated by SDS-PAGE.

2.9.2.2. *Agonist-dependent cholera toxin ribosylation of a $G_{i/o}$ type protein*

Membranes extracted from untreated cells were CT ribosylated in the absence of guanine nucleotide as previously described (Milligan *et al.* 1991; Iiri *et al.* 1989). Cell membranes were washed with 0.15 M potassium phosphate buffer and collected by centrifugation at 10,000 *g* for 5 minutes. 60 μg membrane protein was incubated with 50 μg/ml CT in 0.25 mM potassium phosphate buffer, 20 mM thymidine, 1 mM ATP, 20 mM arginine-HCl, 3 μM [^{32}P]-NAD (50 - 100 μCi/ml) with and without the presence of IL-1α (1 or 10 μg/ml), TNFα (1 or 10 μg/ml) or endothelin-1 (3 μM), in a final volume of 100 μl. The inclusion of 2.5 mM $MgCl_2$ in the reaction mixture was also

tried. After incubating for 1 hour at 30°C, the reaction was terminated as above and the proteins separated by SDS-PAGE.

2.10. SODIUM DODECYL SULPHATE-POLYACYRLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecular size. SDS-PAGE was carried out essentially as described by Laemmli (1970). Buffers used for SDS-PAGE are detailed in appendix V.

2.10.1. Separation of membrane proteins after ADP-ribosylation

Membrane proteins were separated by SDS-PAGE using the Protean II xi gel system (Bio-Rad). Each 20 cm x 16 cm x 1 mm gel was prepared from stock solutions as described below;

12.5% (w/v) acrylamide resolving gel;

Protogel (30% (w/v) acrylamide and 0.8% (w/v) Bisacrylamide) (National Diagnostics, Aylesbury, UK)	12.5 ml
1.5 M TRIS-HCl, pH 8.8	7.2 ml
10% SDS solution	0.3 ml
10% ammonium persulphate	150 µl
double-distilled water	9.85 ml
N, N, N', N', tetramethylethylene-diamine (TEMED) (Sigma)	15 µl

5% (w/v) acrylamide stacking gel

Protogel	1.6 ml
0.5 M TRIS-HCl, pH 6.8	2.5 ml
10% SDS solution	0.1 ml
10% ammonium persulphate	50 µl
double-distilled water	5.75 ml

TEMED 10 µl

The resolving gel was poured and overlaid with a layer of 50% methanol in distilled water. After 1 hour the methanol was removed and the top of the gel rinsed with water. The stacking gel was poured on top of the resolving gel and set with a 15 lane comb. The stacking gel was left 1 hour to polymerise.

Membrane samples were solubilized by boiling for 5 minutes in 30 µl of 1x sample buffer, which contained 2-mercaptoethanol to reduce protein disulphide bonds. Samples were left to cool prior to loading. A prestained molecular weight standard (Gibco) was included on each gel. Samples and standard were not loaded in the outer two wells of any gel and all empty wells were loaded with an equivalent volume of 1x sample buffer to ensure that the samples ran straight. Gels were run overnight (13 hours) at a constant current of 10 mA per gel using a Bio-Rad powerpack at room temperature and tap water was circulated through the cooling system during the run to prevent overheating of the gels. The electrophoresis buffer was stirred throughout by placing the tank on a magnetic stirrer.

Gels were stained for protein by gently agitating in 0.1% coomassie blue stain for 1 hour at room temperature. Gels was then transferred into destain solution, which was changed regularly until the blue bands of protein were visible on a clear background of gel. The sensitivity of coomassie blue staining is 0.3 - 1 µg protein/band. Gels were stained to assess equal loading of protein across the lanes. After staining, the gels were placed between a sheet of filter paper and a sheet of clear cellophane and dried for 2 hours at 80°C using a Bio-Rad gel dryer. The dried gels were exposed to Kodak x-omat x-ray film for approximately 2 days at -70°C with intensifying screens.

2.10.2. Separation of cell proteins for iNOS detection

MC in 25 cm^2 flasks were extracted for iNOS protein by scraping the cell monolayer into 0.5 ml of 1x sample buffer containing 20 mM EDTA and 5 µg/ml carboxypeptidase inhibitor (Sigma). Samples were immediately boiled for 5 minutes, then stored in 100 µl aliquots at -20°C until required.

Two 10 cm x 8 cm x 1 mm minigels were prepared from the following stock solutions;

	7% (w/v) acrylamide resolving gel:	*5% (w/v) acrylamide stacking gel:*
Protogel (30% acrylamide, 0.8% bisacrylamide)	3.5 ml	1.67 ml
1.5 M TRIS-HCl, pH 8.8	5.6 ml	-
0.5 M TRIS-HCl, pH 6.8	-	1.25 ml
10% SDS solution	0.15 ml	0.1 ml
10% ammonium persulphate	0.1 ml	0.05 ml
double-distilled water	5.85 ml	6 ml
TEMED	10 µl	10 µl

Samples were thawed and centrifuged at 12,000 *g* for 2 minutes prior to loading. 20 µl of sample was loaded per lane (~20 µg) and a prestained molecular weight marker was included on each gel. Gels were run at room temperature at 100 volts, until the bromophenol blue tracking dye entered the resolving gel. The voltage was then increased to 150 volts. Gels were run until the bromophenol blue band had reached the bottom of the resolving gel. Gels were then placed into transfer buffer in preparation for western blotting.

2.11. WESTERN BLOTTING

2.11.1. Electrophoretic transfer of proteins to nitrocellulose

Proteins separated on 7% polyacrylamide gels were transferred to nitrocellulose paper using a wet transfer method. The polyacrylamide gel was soaked for 15 minutes in transfer buffer and the lanes of the stacking gel removed. Two pieces of filter paper, a piece of nitrocellulose paper and two nylon pads were also soaked in transfer buffer. The gel was overlaid with a piece of wet nitrocellulose membrane (Protran BA 85) (Schleicher and Schuell, Dassel, Germany) and surrounded on each side by a layer of

filter paper and a nylon pad. The gel 'sandwich' was enclosed in a cassette and placed in the transfer tank (Bio-Rad) with the nitrocellulose nearest the anode. The tank was filled with cold (4°C) transfer buffer and placed on a magnetic stirrer. Electrophoretic transfer was carried out for 35 minutes at 75 volts.

2.11.2. Immunoblotting of nitrocellulose-bound protein

Western blot analysis of iNOS protein was performed according to the method of Dr J. Weidner, MERCK research laboratories, Rahway, NJ (personal communication). N053 rabbit antisera directed against the C-terminal sequence of human iNOS and control N054 blocking peptide were gifts from MERCK. Antisera was diluted 1:1 in glycerol and stored at -20°C for daily use.

Nitrocellulose membranes were incubated for 30 minutes in 3% milk protein (Sigma) in PBS. Membranes were washed once with PBS-T (PBS + 0.05% tween) for 10 minutes, and incubated for 1 hour with a 1:40,000 dilution of iNOS antisera diluted in PBS-T. Membranes were washed 3 x 10 minutes with PBS-T and incubated for 1 hour with sheep polyclonal anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma, A-0407) diluted 1:1000 in PBS-T. Membranes were washed 3 x 10 minutes with PBS, then incubated for 3 - 10 minutes with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (2 x 0.2 g tablets diluted in 20 ml distilled water immediately prior to use) (Sigma, B-5655). When the desired development of bands was reached, the reaction was stopped by transferring the membrane into 4 mM EDTA in PBS, pH 7.4 and incubating for 3-5 minutes. To avoid carryover, all incubations except the wash steps were performed in separate containers.

Specificity of the iNOS antisera was confirmed by adding 50 nM of a blocking peptide, to the working dilution of antisera. The blocking peptide corresponded to the C-terminal sequence of human iNOS, against which the antibody was directed.

Separation of MC samples by SDS-PAGE for analysis of iNOS protein and Western blotting was kindly performed by Dr Christine Murphy (Dept. of Pharmacology, University of Bath).

2.12. NUCLEAR EXTRACTION

Nuclear extracts were prepared from MC for analysis of NF-κB activity. Quiescent MC grown in 25 cm^2 flasks were pretreated with dibutyryl-cAMP (db-cAMP) (100 μM) or media alone for 5 hours, prior to the addition of IL-1α (3 ng/ml) to the media for 30 minutes, 1, 2 or 6 hours. Supernatants were removed and nuclear extracts prepared as previously described (Stylianou *et al.* 1992). All buffers used for the nuclear extraction contained 0.5 mM PMSF. Cells were briefly washed in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and lysed by incubating in 1.5 ml hypotonic buffer containing 0.1% Nonidet P-40 for 10 minutes on ice. Cells were scraped from the flasks and transferred to 1.5 ml eppendorfs. Lysates were centrifuged at 10,000 *g* for 10 minutes at 4°C and the supernatant discarded. The pelleted nuclei were resuspended in 45 μl of lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol) and incubated at 4°C for 15 minutes. Lysed nuclei were vortexed and centrifuged at 10,000 *g* for 10 minutes at 4°C. The supernatant was removed and mixed with 105 μl of storage buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM DTT). Pellets were discarded. The protein content of the samples was estimated, as described in section 2.3. and samples were frozen at -70°C.

2.13. ELECTROPHORETIC MOBILITY SHIFT ASSAY

MC nuclear extracts were analysed for NF-κB activity by electrophoresis, as previously described (O'Neill *et al.* 1992). 4 μg of nuclear extract protein was incubated for 30 minutes at room temperature with 2 x 10^3 cpm ^{32}P-labelled DNA fragment derived from the 5' flanking region of the human serum amyloid A gene (SAA), genomic clone $SAAg^9$, which contains the NF-κB binding site. All reactions were performed in the presence of 3 μg of poly(dI-dC) as nonspecific competitor DNA. Protein-DNA complexes were separated by electrophoresis in 4% acrylamide gels and detected by autoradiography.

Gel mobility shift assays were kindly performed by Dr Luke O'Neill (Trinity College, Dublin).

2.14. STATISTICAL ANALYSIS

The n number for each experiment is given in the respective figure legend and refers to the number of MC cultures tested, which were derived from different donors.

Statistical analysis was performed on peptide data which had been $\log_{10}$ transformed to make the data more normally distributed. The distribution of data around the normal was checked before and after transformation using the homogeneity of variance test. Groups of data were then analysed by two-way analysis of variance (ANOVA) to determine if any statistical differences existed within the data group. Data which was significantly different at the $p<0.05$ level was further compared using Dunnetts t-test for the comparison of multiple groups to a control (Dunnett, 1964). This latter test identified which treatments within the group were significantly different from the control. The levels of significance achieved using Dunnetts t-test are denoted by * = $p<0.05$ and ** = $p<0.01$.

Two-way ANOVA and homogeneity of variance tests were performed using the software package, Mini-tab for windows.

3. CHARACTERIZATION OF THE KINETICS AND SPECIFICITY OF IL-6, IL-8, MCP-1 AND RANTES EXPRESSION AND PRODUCTION IN HUMAN MESANGIAL CELLS

3.1. RATIONALE FOR STUDY

It has been previously shown that human mesangial cells in culture have the potential to secrete IL-6, IL-8 and MCP-1 following IL-1 or TNF stimulation (Brown *et al.* 1991b; Abbott *et al.* 1991; Brown *et al.* 1992; Rovin *et al.* 1992). To investigate the regulation of these cytokines in MC, initial experiments were carried out to determine the time courses of gene expression following IL-1 or TNF activation, and the corresponding dose-response curves. This enabled appropriate stimulation times and sub-maximal doses of agonist to be selected in later experiments. Furthermore the potential of human MC to express the C-C chemokine RANTES, the selective chemotactic factor for CD45RO/CD4$^+$ T cells and monocytes was investigated.

3.2. RESULTS

In the following results section, representative figures have been used to demonstrate the profiles of IL-6, IL-8, MCP-1 and RANTES mRNA expression and peptide production in IL-1α or TNFα activated MC. While the profiles of peptide generation were found to be similar across MC isolated from different donors, some variation in the levels of peptide production were observed (in general, up to 5 fold differences). Unless otherwise stated, all experiments were carried out on growth-arrested MC, in the absence of FCS. Cells were growth-arrested for 24 hours prior to the start of the experiments.

3.2.1. IL-6 mRNA expression and peptide production by cytokine activated mesangial cells

MC incubated with media alone for 5, 24 or 48 hours did not express detectable IL-6 mRNA transcripts. Stimulation of MC with IL-1α (3 ng/ml) resulted in time-dependent IL-6 mRNA expression (Fig. 18). Transcripts were first detected 1 to 2 hours post-stimulation, maximal expression occurred between 8 and 16 hours and mRNA levels had declined to 25 ± 8% (mean ± SEM, n=4) of the peak levels by 48 hours.

IL-6 peptide was not detected in culture supernatants from MC incubated with media alone for 5, 24 or 48 hours (<0.03 ng/ml). IL-6 peptide was first detected in culture supernatants 8 hours after IL-1α stimulation (0.1 ± 0.02 ng/ml (mean ± SEM)) and levels were still increasing at 48 hours (Fig. 19). Production of IL-6 at 48 hours was 24 ± 1 ng/ml (mean ± SEM). Dose-response studies demonstrate stimulation with 3 ng/ml IL-1α resulted in sub-maximal release of IL-6 peptide after 18 hours (Fig. 20a). This dose of IL-1α was used in future experiments.

In comparison to IL-1α, TNFα was found to be a very poor stimulus for IL-6 production in MC. Stimulation with 30 ng/ml TNFα resulted in IL-6 peptide levels of 0.09 ± 0.03 ng/ml (mean ± SEM) after 18 hours (Fig. 20b). This compares to 6.6 ± 0.1 ng/ml (mean ± SEM) of IL-6 peptide measured after stimulation with 30 ng/ml IL-1α for 18 hours (Fig. 20a). The time course of TNFα stimulated IL-6 mRNA expression and peptide production were not investigated during this study, as TNFα was such an ineffective stimulus for IL-6 production under the experimental conditions employed. Interestingly, the TNFα response could be improved if the experimental culture conditions were changed. To enable the experiments to be performed in a 'defined' system, all studies were carried out in the absence of serum, on quiescent MC which had been pre-incubated in serum-free media for 24 hours prior to the start of the experiment. A 4 fold increase in IL-6 peptide production after 48 hours was however observed if MC were not growth-arrested prior to TNFα stimulation, and a 13 fold increase in IL-6 production was observed if cells were stimulated in the presence of 2% serum (Fig. 21).

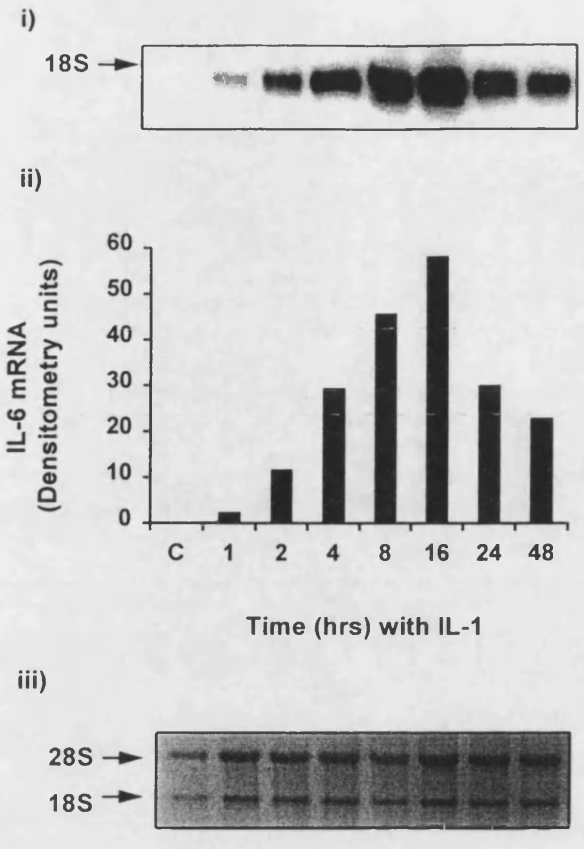


Figure 18. Time-dependent expression of IL-6 mRNA in IL-1 activated MC. MC cultured in 25 cm^2 flasks were stimulated with 3 ng/ml IL-1α for the times indicated. Control cells (C) were treated with media alone for 24 hours. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=5.

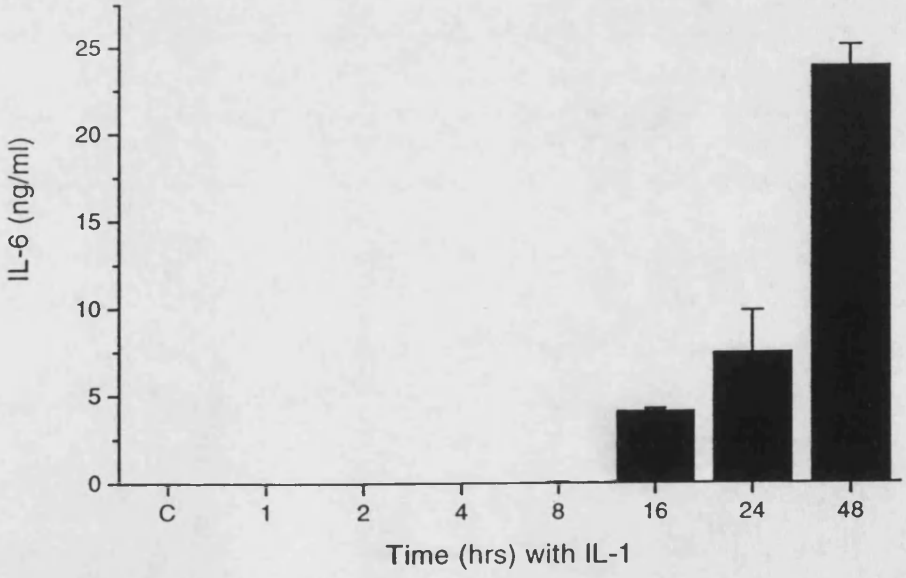
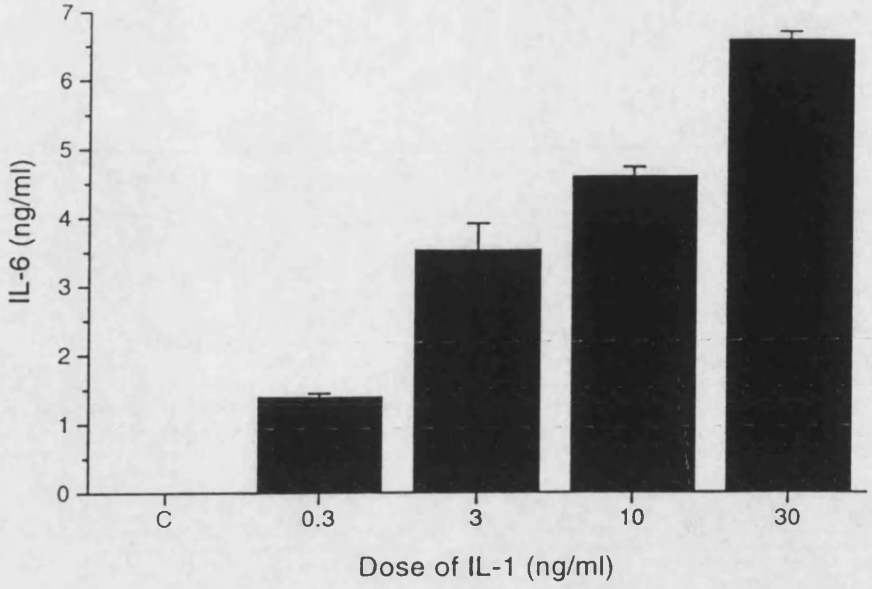


Figure 19. Time course of IL-6 peptide production by IL-1 stimulated MC. MC cultured in 24 multi-well plates were stimulated with 1 ml of 3 ng/ml IL-1α for the times indicated. Control wells (C) were treated with media alone for 5 to 48 hours. Cell supernatants were quantitated for extracellular IL-6 peptide by ELISA. Results are the mean ± SEM of duplicate samples from a representative experiment, similar results were obtained in 4 other experiments.

a)



b)

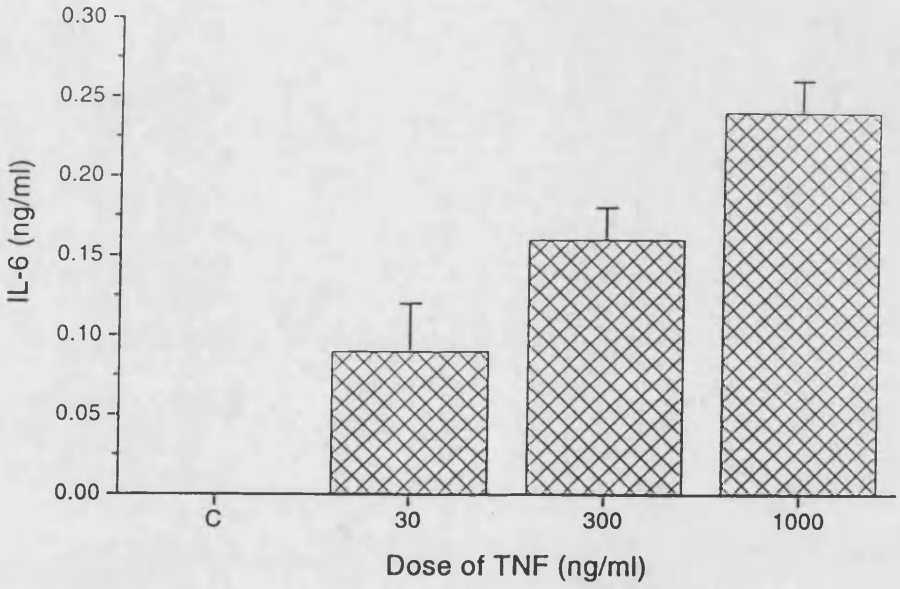


Figure 20. Dose-dependent production of IL-6 peptide by IL-1 or TNF treated MC. MC cultured in 24 multi-well plates were stimulated with increasing doses of IL-1α (a) or TNFα (b) for 18 hours. Control wells (C) were treated with media alone for 18 hours. Cell supernatants were quantitated for extracellular IL-6 peptide by ELISA. Results are the mean ± SEM of duplicate samples from a representative experiment, similar results were obtained in 4 other experiments.

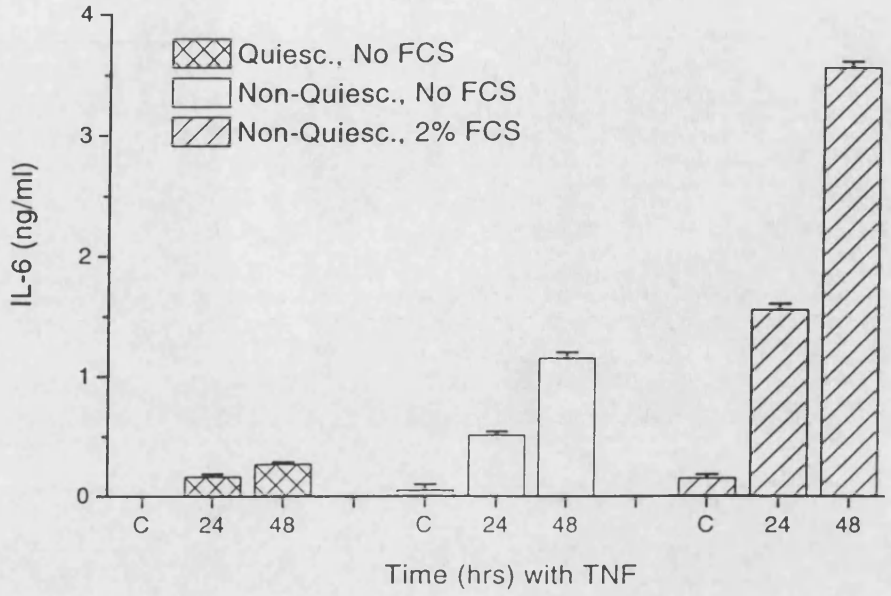


Figure 21. Effect of different culture conditions on TNF induced IL-6 peptide production by MC. MC cultured in 24 multi-well plates were either preincubated in serum free medium for 24 hours, or left in 'complete' Waymouths medium prior to stimulation with 300 ng/ml of TNFα for 18 hours, in both the absence and presence of 2% FCS. IL-6 peptide levels of cell supernatants were quantitated by ELISA. Results are the mean ± SEM of duplicate samples, n=1.

3.2.2. IL-8 mRNA expression and peptide production by cytokine activated mesangial cells

MC incubated in media alone for 5, 24 or 48 hours did not express detectable IL-8 mRNA transcripts. Stimulation with IL-1α (3 ng/ml) resulted in IL-8 mRNA expression which was detected 1 hour post-stimulation (Fig. 22a), maximal expression occurred at 8 hours and mRNA levels had declined to 20 ± 8% (mean ± SEM, n=3) of the peak levels by 48 hours. Similarly, stimulation with TNFα (300 ng/ml) induced IL-8 mRNA expression which was detected 1 hour post-stimulation and maximal expression had occurred at 8 hours (Fig. 22b). However, in contrast to IL-1α, the TNFα response was more sustained, as mRNA levels remained elevated at 58 ± 20% (mean ± SEM, n=3) of the peak levels at 72 hours.

IL-8 peptide was not detected in culture supernatants from MC incubated with media alone for 5, 24 or 48 hours (<0.05 ng/ml). IL-8 peptide was first detected in culture supernatants 4 hours after IL-1α stimulation (3.5 ± 0.3 ng/ml (mean ± SEM)) and continued to increase upto 24 hours where levels stabilized (Fig. 23). Mean levels of IL-8 at 24 hours were 69 ± 1 ng/ml (mean ± SEM). Previous work carried out in this laboratory showed the time course of TNFα induced IL-8 production was similar to IL-1α, when measured upto 24 hours post-stimulation (Brown *et al.* 1991b).

Dose-response studies demonstrated IL-8 peptide production was dose-related over the concentration range of IL-1α (0.3 - 30 ng/ml) and TNFα (30 - 300 ng/ml) employed (Fig. 24a and b). Sub-optimal doses of IL-1α (3 ng/ml) and TNFα (300 ng/ml) were used in all further experiments. TNFα was found to be approximately 300 fold less potent than IL-1α in inducing IL-8 peptide production after 18 hours.

3.2.3. MCP-1 mRNA expression by cytokine activated mesangial cells

MC incubated in media alone for 5, 24, 48 or 72 hours did not express detectable MCP-1 mRNA transcripts. Stimulation of MC with IL-1α (3 ng/ml) resulted in MCP-1 mRNA expression which was detected 1 hour post-stimulation, maximal expression occurred at 8 hours and mRNA levels declined to 30 ± 15% (mean ± SEM, n=4) of the peak levels by 48 hours (Fig. 25a). Stimulation with TNFα (300 ng/ml) induced MCP-

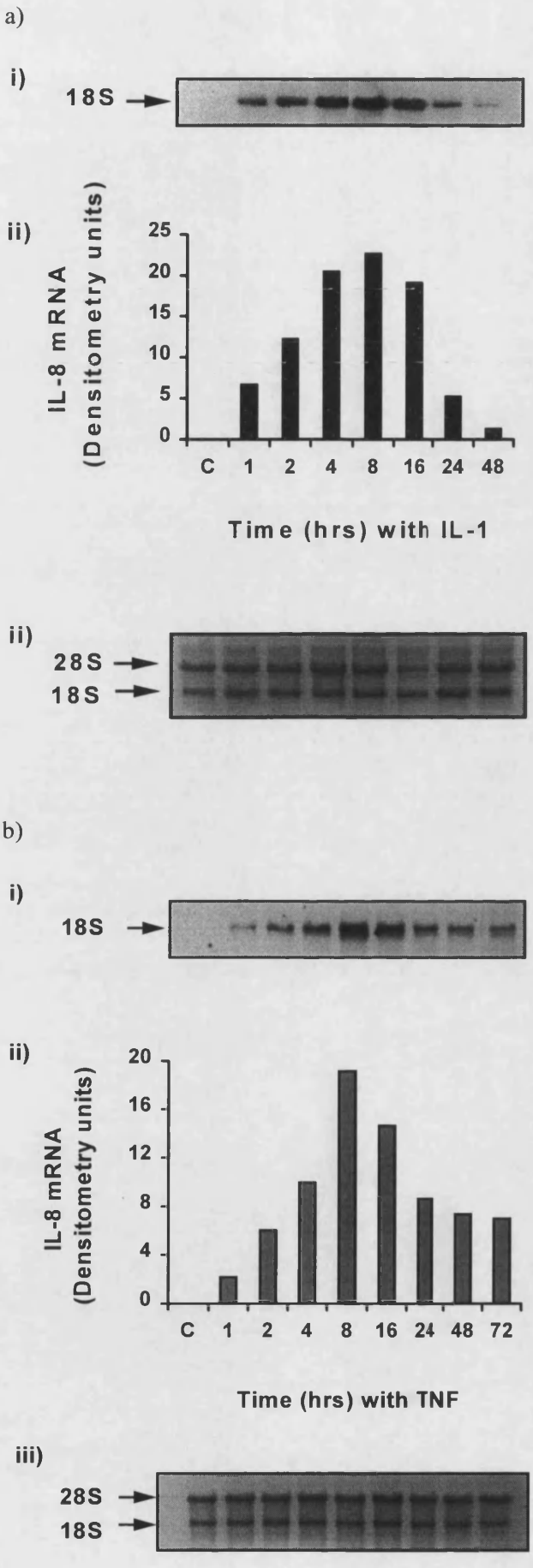


Figure 22. Time-dependent expression of IL-8 mRNA by IL-1 or TNF treated MC. MC culture in 25 cm^2 flasks were stimulated with either 3 ng/ml IL-1α (a) or 300 ng/ml TNFα (b) for the times indicated. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=3. Similar results were obtained in 2 other experiments.

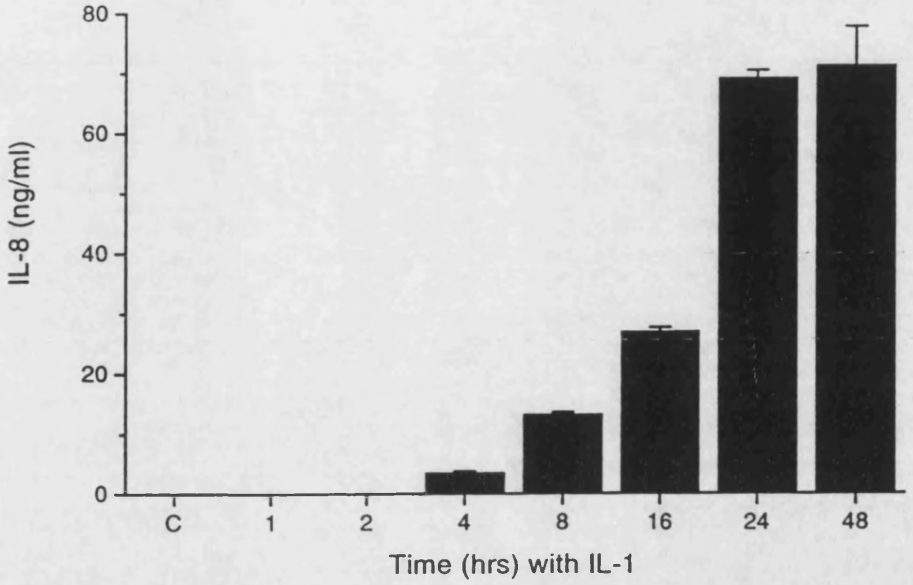
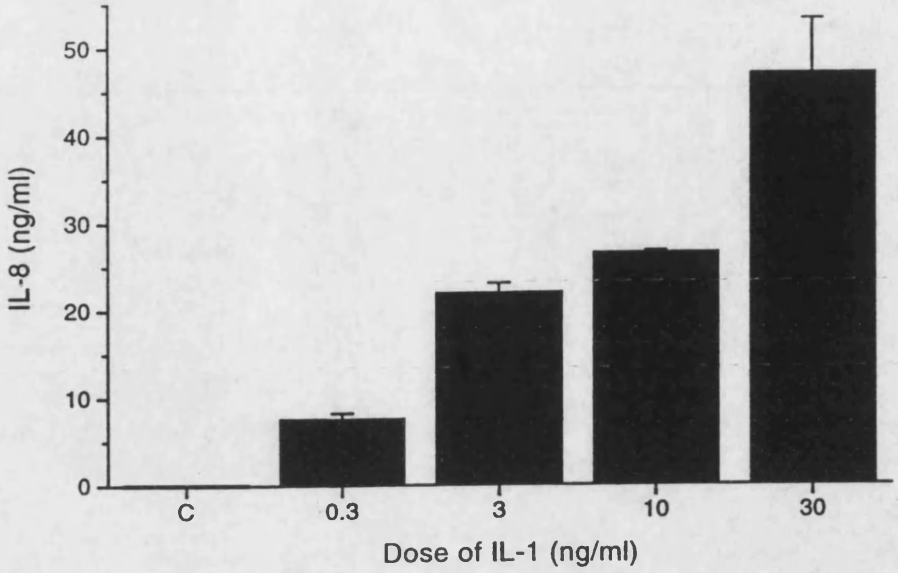


Figure 23. Time course of IL-8 peptide production by IL-1 activated MC. MC cultured in 24 multi-well plates were stimulated with 1 ml of 3 ng/ml IL-1α for the times indicated. Cell supernatants were quantitated for extracellular IL-8 peptide by ELISA. Results are the mean ± SEM of duplicate samples from a representative experiment, similar results were obtained in 1 other experiment.

a)



b)

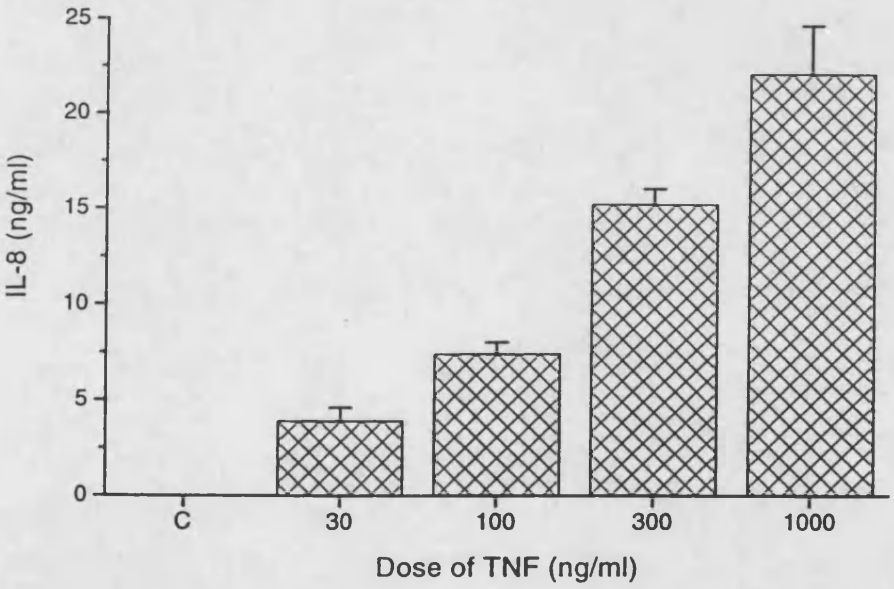


Figure 24. Dose-dependent production of IL-8 peptide by IL-1 or TNF stimulated MC. MC cultured in 24 multi-well plates were stimulated with increasing doses of either IL-1α (a) or TNFα (b) for 18 hours. IL-8 peptide levels of cell supernatants were quantitated by ELISA. Results are the mean ± SEM of duplicate samples from a representative experiment, similar results were obtained in 5 other experiments.

1 mRNA expression with similar kinetics to that observed following IL-1α stimulation over the first 8 hours (Fig. 25b). Interestingly however, the TNFα induced MCP-1 mRNA expression was far more sustained than the IL-1α response, with mRNA levels remaining elevated at near maximal levels for upto 72 hours. MCP-1 mRNA expression was 86 ± 7% (mean ± SEM, n=3) of the peak levels at 72 hours.

3.2.4. RANTES mRNA expression and peptide production by cytokine activated mesangial cells

In addition to IL-8 and MCP-1, cytokine activated MC have the ability to secrete RANTES, another member of the C-C chemokine family. The gene expression and production of RANTES was investigated following either IL-1α, TNFα or LPS activation of MC.

MC incubated in media alone for 4, 24, 48 or 72 hours did not express detectable RANTES mRNA transcripts. Stimulation of MC with either IL-1α (3 ng/ml) or TNFα (300 ng/ml) induced time-dependent RANTES mRNA expression. IL-1α induced RANTES mRNA transcripts by 2 hours post-stimulation, expression was maximal at 24 hours and declined to 28 ± 6% (mean ± SEM, n=4) of the peak levels at 72 hours (Fig. 26a). In marked contrast, TNFα induced a later time course of RANTES mRNA expression and the response was more sustained. mRNA transcripts were first detected 4-6 hours post-stimulation, maximal increase in gene expression occurred at 48 hours and levels remained elevated at 72 hours (90 ± 7% (mean ± SEM, n=4) of the peak levels) (Fig. 26b).

Supernatants from MC incubated with media alone for 4, 24, 48, or 72 hours contained no detectable RANTES peptide (<0.06 ng/ml). Stimulation with IL-1α (3 ng/ml) induced production of RANTES peptide which was first detected in culture supernatants 8 hours post-stimulation (0.6 ± 0.1 ng/ml (mean ± SEM)) and continued to rise upto 48 hours, where levels stabilized (Fig. 27a). Peak levels of RANTES produced at 48 hours were 1.4 ± 0.3 ng/ml (mean ± SEM). In comparison, TNFα induced detectable levels of RANTES peptide by 16 hours post-stimulation (1.0 ± 0.2 ng/ml (mean ± SEM)) and

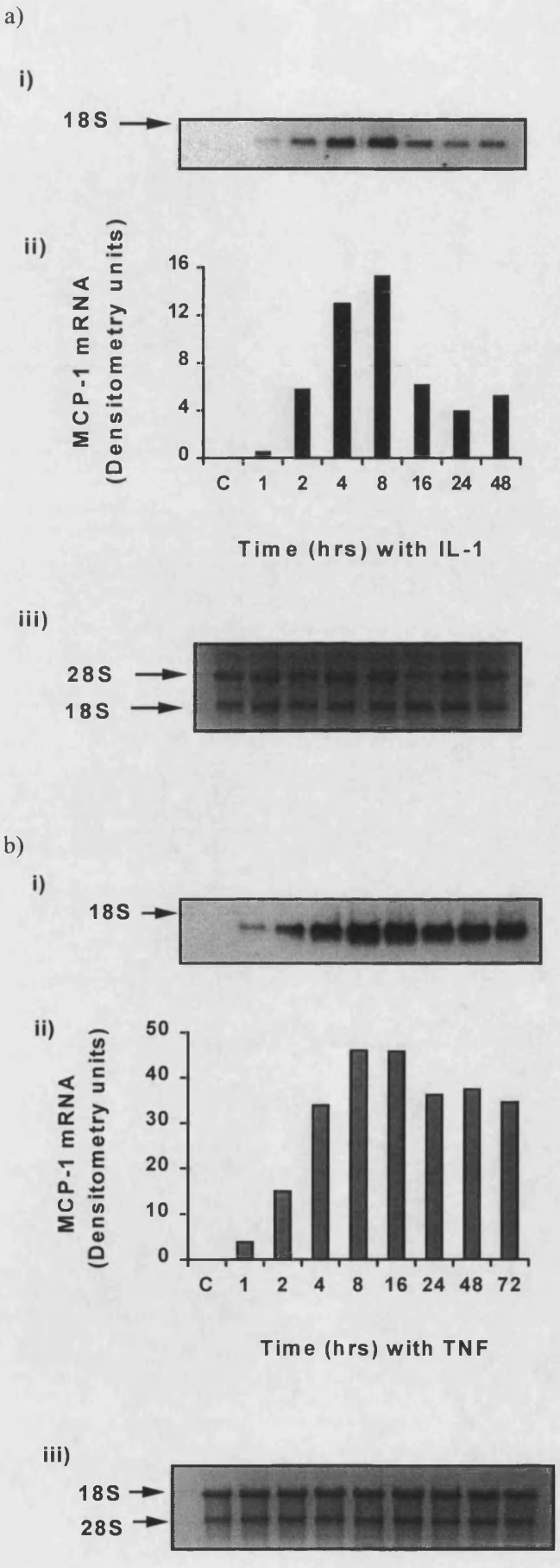


Figure 25. Time-dependent expression of MCP-1 mRNA by IL-1 or TNF activated MC. MC cultured in 25 cm^2 flasks were stimulated with either 3 ng/ml IL-1α (a) or 300 ng/ml TNFα (b) for the times indicated. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=4 and 3 for the IL-1α (a) and TNFα (b) data respectively.

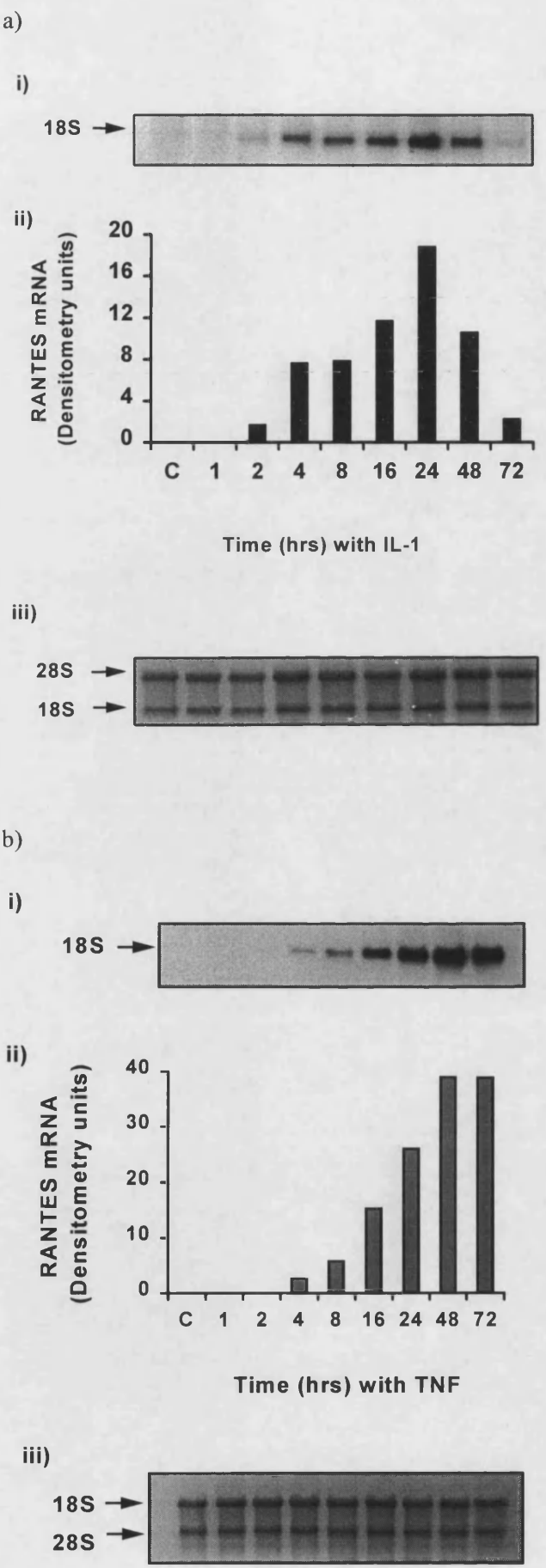


Figure 26. Time-dependent expression of RANTES mRNA by IL-1 or TNF activated MC. MC cultured in 25 cm^2 flasks were stimulated with either 3 ng/ml IL-1α (a) or 300 ng/ml TNFα (b) for the times indicated. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=4.

levels were still increasing at 96 hours (Fig. 27b). Mean production of RANTES at 96 hours was 86 ± 10 ng/ml (mean ± SEM). Dose-response studies demonstrate that stimulation with 3 ng/ml IL-1α resulted in maximal release of RANTES peptide, no further increase in production was observed at the higher doses of IL-1α (Fig. 28a). In contrast, TNFα stimulated RANTES peptide production was dose-related between 30 - 1000 ng/ml, the concentration range investigated (Fig. 28b). Concentrations of 1 ng/ml IL-1α and 50 ng/ml TNFα were used in later experiments, when sub-optimal doses were required. The time course and dose-response data on RANTES peptide production indicate that in marked contrast to TNFα, IL-1α is a poor stimulus for RANTES production in MC.

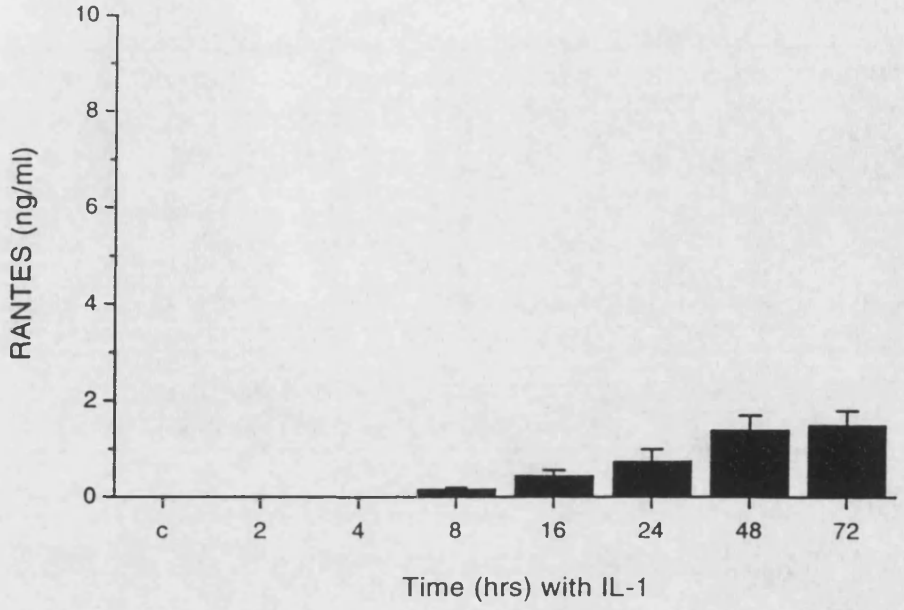
Stimulation of MC with LPS (0.1 - 10 μg/ml) resulted in undetectable (<0.06 ng/ml) levels of RANTES peptide in culture supernatants after 48 hours (n=2, data not shown).

3.2.5. Investigation of a secondary protein involvement in IL-1 or TNF induced chemokine gene expression

Comparison of the kinetics of RANTES, IL-8 and MCP-1 gene expression in MC shows the time course of induction of RANTES mRNA following either IL-1α or TNFα stimulation was much later than IL-8 or MCP-1 (Fig. 29). Both IL-8 and MCP-1 were maximally expressed 8 hours post stimulation, while RANTES mRNA expression was only maximal 1 and 2 days after IL-1α and TNFα stimulation respectively.

To investigate whether the IL-1α or TNFα regulation of RANTES mRNA expression was dependent on *de novo* protein synthesis, MC were pretreated for 1 hour with the protein synthesis inhibitor cycloheximide (CHX) (5 μg/ml) prior to cytokine stimulation. CHX treatment resulted in superinduction of IL-1α (3 ng/ml) induced RANTES mRNA transcripts after 8 and 24 hours (Fig. 30a), indicating the IL-1α response was not protein synthesis dependent. In marked contrast, CHX inhibited TNFα (300 ng/ml) induced RANTES mRNA expression by 79.4 ± 6.4% (mean ± SEM, n=4) at 48 hours (Fig. 30b). The inhibitory effect of CHX on the TNFα response was not due to a toxic effect of the drug treatment as cell viability, assessed by trypan blue exclusion, was >90% after treatment. Furthermore, similar levels of inhibition were

a)



b)

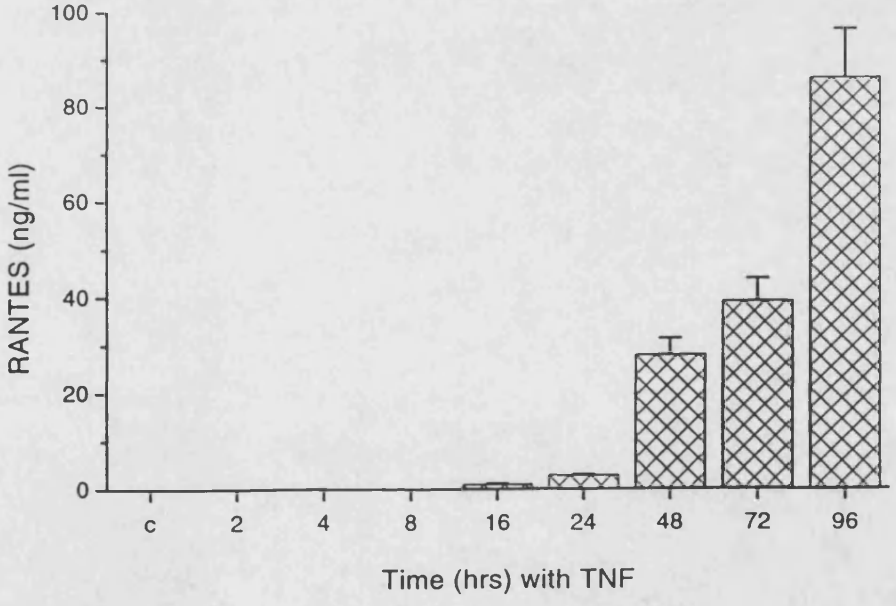
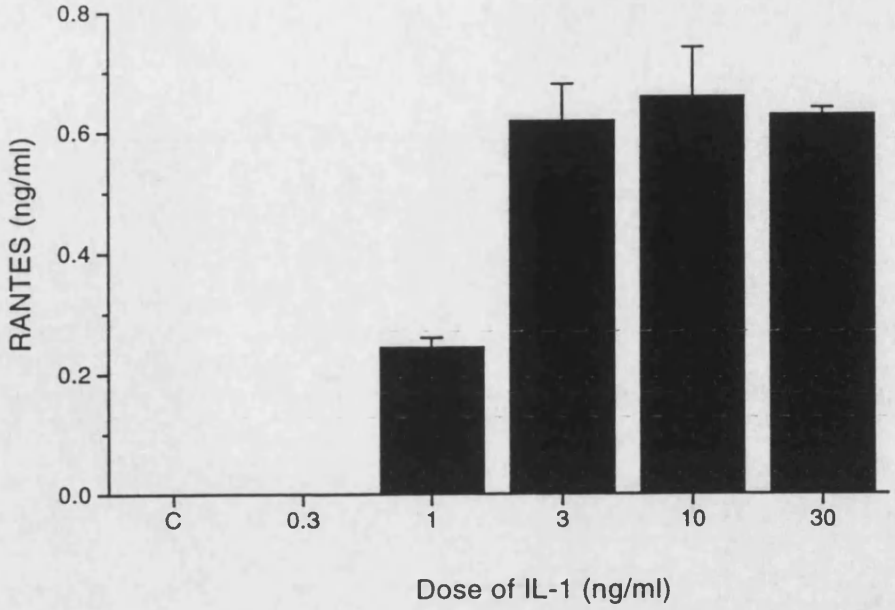


Figure 27. Time course of RANTES peptide production by IL-1 or TNF activated MC. MC cultured in 24 multi-well plates were stimulated with either 3 ng/ml IL-1α (a) or 300 ng/ml TNFα (b) for the times indicated. Cell supernatants were quantitated for extracellular RANTES peptide by ELISA. Results are the mean ± SEM of duplicate samples from a representative experiment, similar results were obtained in 4 other experiments.

a)



b)

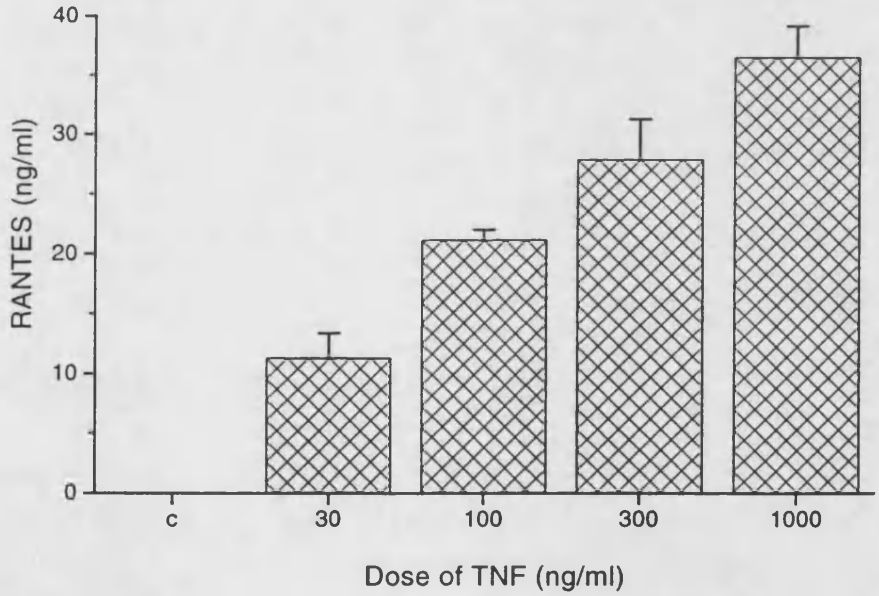


Figure 28. Dose-dependent production of RANTES peptide by IL-1 or TNF activated MC. MC cultured in 24 multi-well plates were stimulated with increasing doses of either IL-1α for 24 hours (a) or TNFα for 48 hours (b). Cell supernatants were quantitated for extracellular RANTES peptide by ELISA. Results are the mean ± SEM of duplicate samples from a representative experiment, similar results were obtained in 4 other experiments.

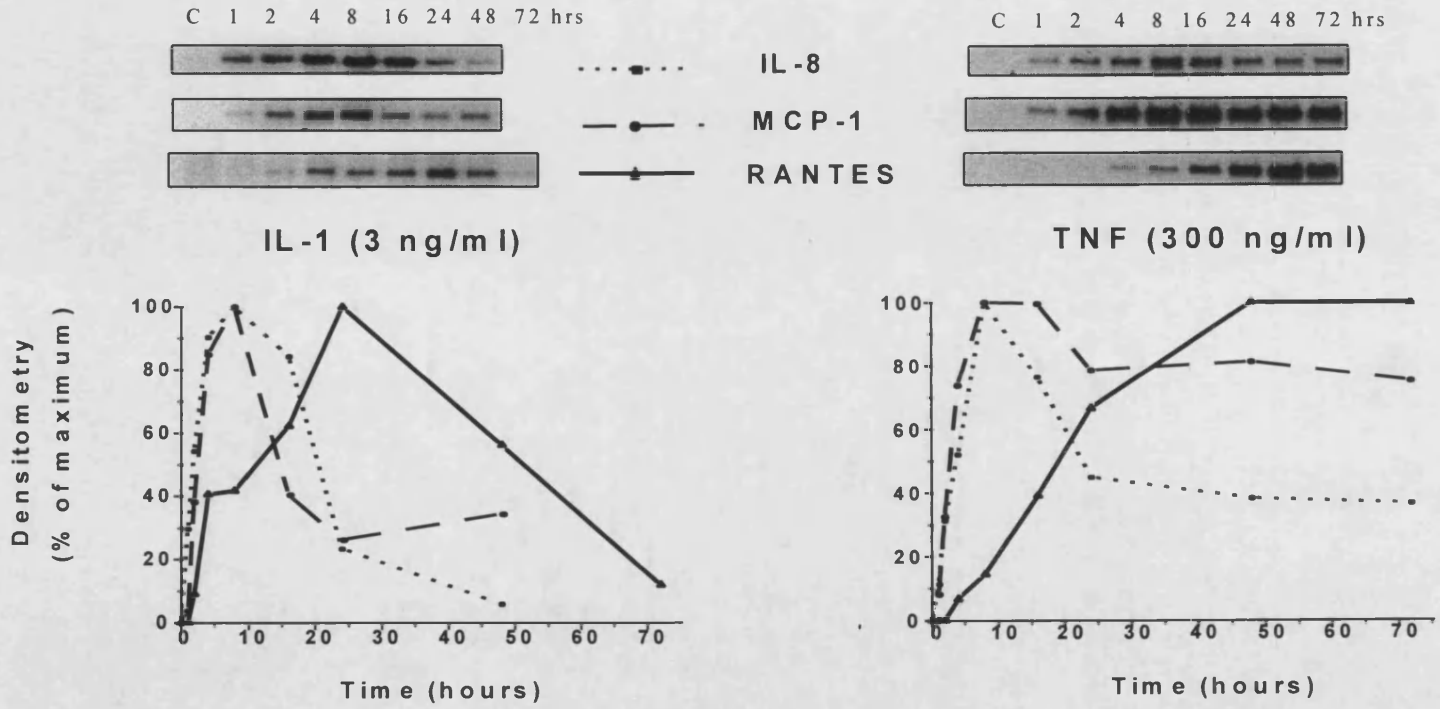


Figure 29. Summary figure of the kinetics of IL-1 and TNF induced chemokine mRNA expression in MC. MC cultured in 25 cm^2 flasks were stimulated with either 3 ng/ml IL-1α or 300 ng/ml TNFα for the times indicated. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs were analysed by densitometry and the results plotted as % of maximal expression. Representative data is shown, n≥3.

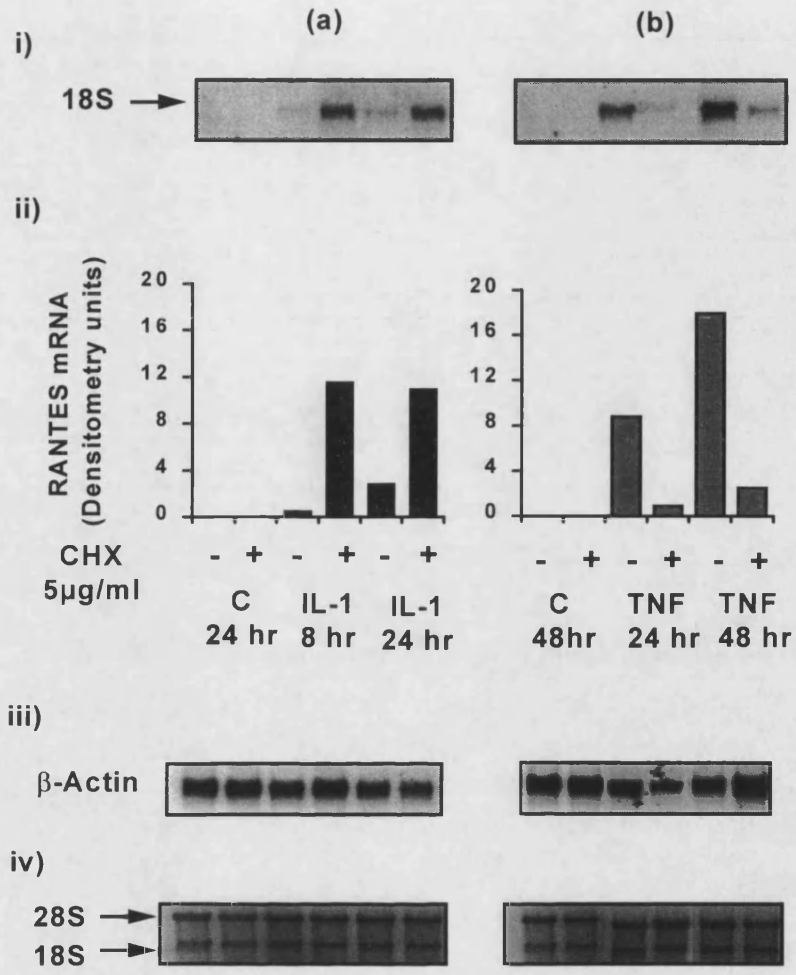


Figure 30. Effect of cycloheximide on either IL-1 or TNF induced RANTES mRNA expression. MC were pretreated with CHX (5µg/ml) for 1 hour prior to stimulation with either 3 ng/ml IL-1α for 8 and 24 hours (a), or stimulation with 300 ng/ml TNFα for 24 and 48 hours (b). Total cellular RNA was extracted and northern analysis carried out. Autoradiographs of RANTES probed membranes (i) were analysed by densitometry and the results plotted using the absorbance units (ii). Identical membranes were probed with the house-keeping gene β-actin (iii) to assess equal loading, along with the ethidium bromide stained gels (iv). Representative blots are shown, n=4.

observed when lower doses of CHX (2.5 μg/ml) and TNFα (50 ng/ml) were employed (Fig. 31). The inhibitory effect of CHX indicates the TNFα induction of RANTES mRNA requires *de-novo* protein synthesis and therefore may involve secondary proteins. Preincubation with 5 μg/ml CHX resulted in ≥90% inhibition of IL-1α and TNFα induced MC protein synthesis, assessed by measuring RANTES peptide levels in cell supernatants (data not shown).

An involvement of secondary proteins in IL-1α or TNFα induced IL-8 and MCP-1 mRNA expression was also investigated. Preliminary data showed CHX (5 μg/ml) treatment resulted in superinduction of IL-1α (3 ng/ml) induced IL-8 (Fig. 32a) and MCP-1 (Fig. 33a) mRNA expression after 24 hours. These results are consistent with previous findings in human MC (Dr Z. Brown, personal communication). Similarly, CHX treatment resulted in superinduction of TNFα induced IL-8 mRNA expression (Fig. 32b). In contrast, only minor potentiation was observed for TNFα induced MCP-1 mRNA expression after 24 hours (Fig. 33b). Together the results indicate that both the IL-1α and TNFα induction of IL-8 and MCP-1 gene expression were not protein synthesis dependent.

CHX treatment had no effect on basal levels of RANTES, IL-8 or MCP-1 mRNA expression (Fig. 30 - 33).

3.2.6. Role of endogenous IL-1 in RANTES peptide production by TNF activated mesangial cells

Cycloheximide treatment inhibited TNFα induced RANTES gene expression indicating the TNFα response requires *de novo* protein synthesis and may involve secondary proteins. Expression of IL-1α by TNFα activated human MC has been previously reported (Abbott *et al.* 1992). To establish if endogenous production of IL-1 played a role in the TNFα induction of RANTES peptide, MC were pretreated for 15 minutes with human recombinant IL-1 receptor antagonist (IL-1ra, 30 to 300 ng/ml) prior to the addition of IL-1α or TNFα for 48 hours. Treatment of MC with IL-1ra at ≥100 ng/ml resulted in complete inhibition of IL-1α induced RANTES peptide production (Fig. 34).

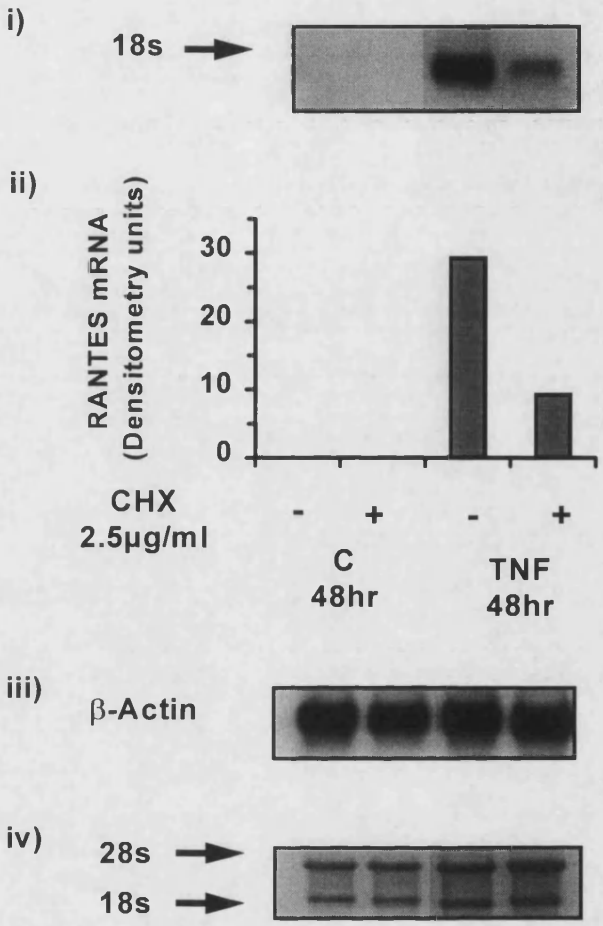


Figure 31. Effect of low doses of cycloheximide and TNF on RANTES mRNA expression. MC were pretreated with 2.5 µg/ml CHX for 1 hours prior to the addition of 50 ng/ml TNFα to the drug-containing media. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs of the RANTES probed membranes (i) were analysed by densitometry and the results plotted using the absorbance units (ii). Identical membranes were probed with the house-keeping gene β-actin (iii) to assess equal loading, along with the ethidium bromide stained gels (iv). n=1.

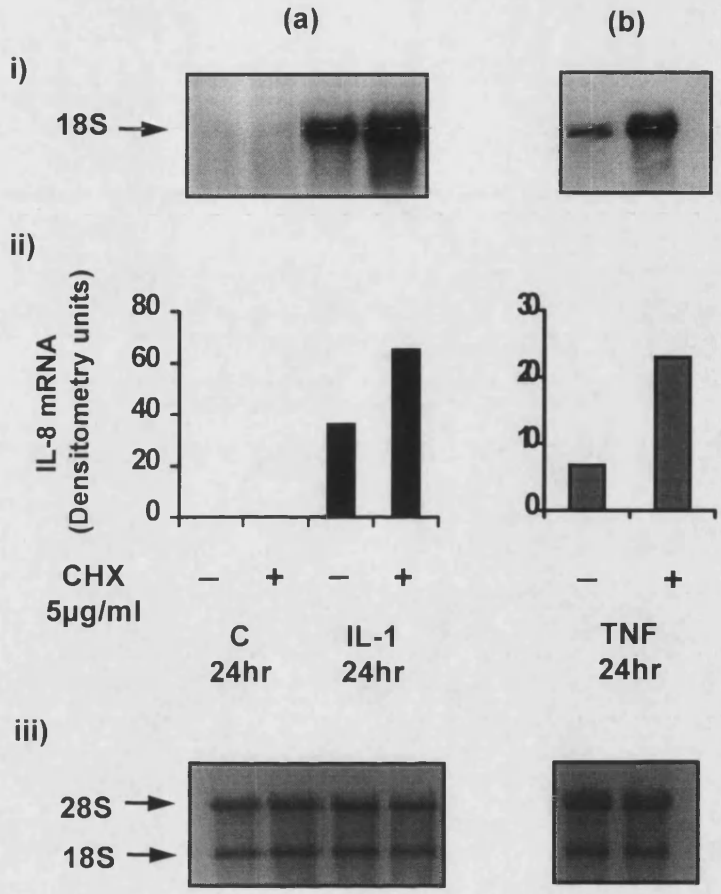


Figure 32. Effect of cycloheximide on IL-1 or TNF induced IL-8 mRNA expression. MC were pretreated with CHX (5 µg/ml) for 1 hour prior to stimulation with either 3 ng/ml IL-1α (a) or 100 ng/ml TNFα for 24 hours (b). Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iv). Representative blots are shown, n=1 and 2 for the IL-1α (a) and TNFα (b) data respectively.

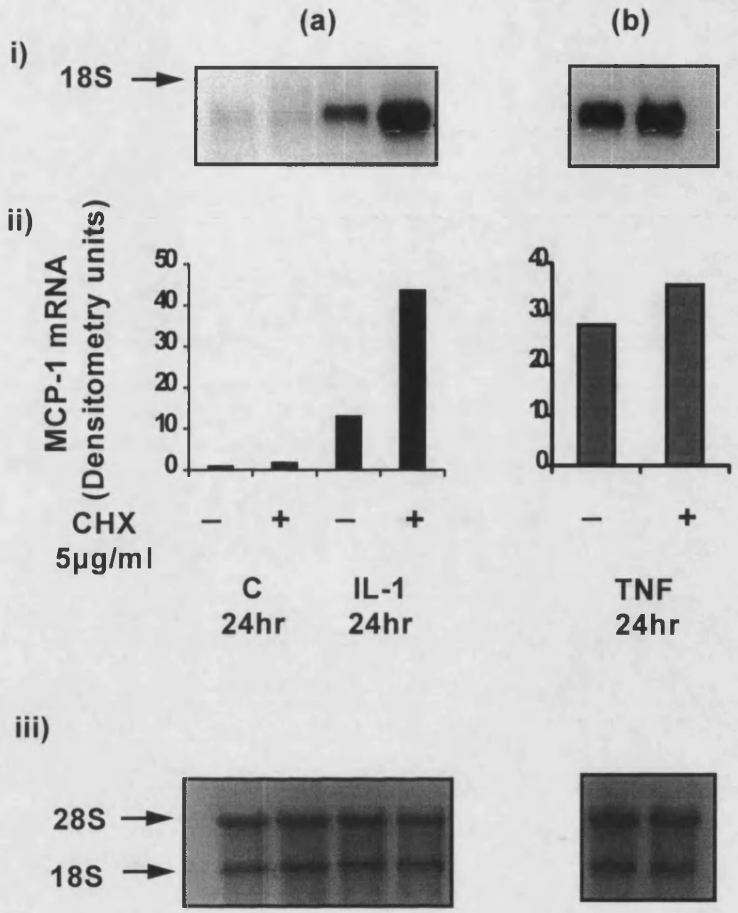


Figure 33. Effect of cycloheximide on IL-1 0r TNF induced MCP-1 mRNA expression. MC were pretreated with CHX (5 µg/ml) for 1 hour prior to stimulation with either 3 ng/ml IL-1α (a) or 100 ng/ml TNFα for 24 hours (b). Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iv). Representative blots are shown, n=1 and 2 for the IL-1α (a) and TNFα (b) data respectively.

In contrast, IL-1ra had no effect on TNFα induced RANTES peptide production (Fig. 34).

3.2.7. Role of secondary protein(s) in the TNF induction of RANTES

To establish if TNFα activation of MC resulted in secretion of secondary protein(s) which were responsible for the induction of RANTES production in the absence of exogenously added TNFα, neutralising antibody experiments were undertaken. MC grown in 24 multi-well tissue culture plates were stimulated for 24 hours with 50 ng/ml of TNFα. Supernatants were collected and premixed for 30 minutes at 37°C with a 1:50 dilution of sheep anti-TNF serum or normal sheep serum. Supernatants were then transferred to fresh cells and incubated for a further 48 hours. Table 7 (a) shows that stimulation of MC for 24 hours with 50 ng/ml of TNFα resulted in 4.8 ± 3.2 ng/ml (mean ± SEM, n=4) of RANTES peptide production. Incubation of fresh MC for 48 hours with supernatants from TNFα stimulated cells in the presence of neutralising amounts of anti-TNF antibody resulted in unaltered RANTES peptide levels (Table 7 (b)), compared with supernatants in the presence of control antibody (normal sheep serum) which resulted in increased production of RANTES to 12.8 ± 3.7 ng/ml (Table 7 (c)). Thus secondary proteins secreted by TNFα stimulated MC were unable to induce RANTES peptide production in the absence of 'active' TNF. The specificity and neutralising ability of the anti-TNF antibody was confirmed by stimulating the cells with TNFα (50 ng/ml) either in the presence of a 1/50 dilution of anti-TNF serum or normal sheep serum (Table 7 (d), (e)). Anti-TNF antibody resulted in 100% inhibition of 50 ng/ml TNF activity (Table 7 (d)).

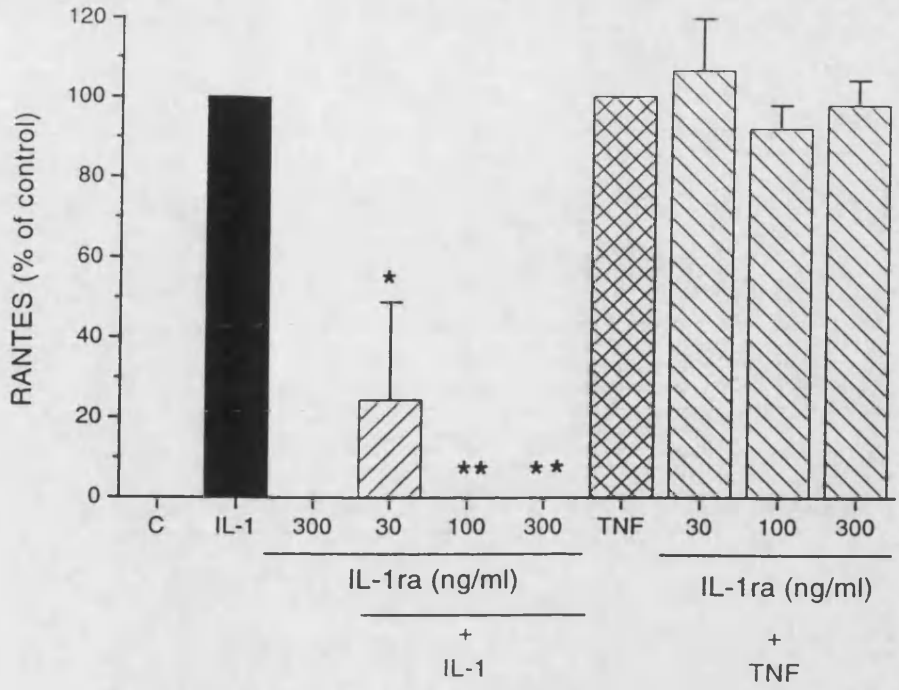


Figure 34. Effect of IL-1 receptor antagonist on IL-1 or TNF stimulated RANTES peptide production. MC cultured in 24 multi-well plates were pretreated with IL-1ra (30 - 300 ng/ml) for 15 minutes prior to the addition of IL-1α (3 ng/ml) or TNFα (300 ng/ml) to the drug-containing media for 48 hours. Cell supernatants were quantitated for extracellular RANTES peptide by ELISA. Results are expressed as a % of the IL-1α or TNFα control and are the mean ± SEM of n=3.

TREATMENT	RANTES (ng/ml) (mean ± SEM)
(A) TNF (50 ng/ml), 24 hr	4.8 ± 3.2
(B) 24 hr supernatant + anti-TNF serum, 48 hr	4.4 ± 3.2
(C) 24 hr supernatant + NSS, 48 hr	12.8 ± 3.7
(D) TNF (50 ng/ml) + anti-TNF serum, 48 hr	<0.06 ± 0
(E) TNF (50 ng/ml) + NSS, 48 hr	8.8 ± 4.0

Table 7. Effect of secondary proteins on RANTES peptide production. MC cultured in 24 multi-well plates were stimulated for 24 hours with 50 ng/ml TNFα (A). Cell supernatants were removed, premixed for 30 minutes with neutralising amounts of anti-TNFα serum, or normal sheep serum and transferred to fresh MC for a further 48 hours (B and C respectively). The ability of a 1:50 dilution of anti-TNFα serum to completely neutralise the activity of 50 ng/ml TNFα was confirmed by stimulating the cells with TNFα premixed with anti-TNFα serum (D) or normal sheep serum (E) for 48 hours. Cell supernatants were quantitated for extracellular RANTES peptide by ELISA. Results are the mean ± SEM of n=4.

3.3. DISCUSSION

The molecular mechanisms controlling leukocyte migration to a site of tissue injury are multistep, and chemokine production by cells of the extravascular site is thought to play an important role in mediating the accumulation of specific leukocyte populations at different phases of the inflammatory response. A number of studies have demonstrated cultured human MC, when activated by cytokines, are a rich source of the neutrophil chemoattractant, IL-8 and the monocyte chemotactic factor, MCP-1 (Brown *et al.* 1991b; Abbott *et al.* 1991; Kusner *et al.* 1991; Brown *et al.* 1992; Rovin *et al.* 1992). This has lead to the proposal that MC may play a key role in the initiation and propagation of inflammatory events within the glomerulus. The results presented in this chapter support this hypothesis and demonstrate that in addition to IL-8 and MCP-1, cytokine-activated human mesangial cells have the ability to secrete RANTES and therefore have the potential to induce the selective attraction of CD45RO/CD4$^+$ T cells into the inflamed glomerulus. The production of these three chemokines by MC was found to be differentially regulated at several levels.

The profile of RANTES mRNA expression induced by IL-1 and TNF was different. TNF induced a later time course of RANTES expression and the signal was more sustained than that observed after IL-1 stimulation. Maximal RANTES mRNA expression was observed 24 and 48 hours post stimulation with either IL-1 or TNF respectively, and while the IL-1 response had declined by 72 hours, TNF induced RANTES mRNA levels remained maximally elevated at 72 hours. Similar time course differences were observed at the level of protein production. IL-1 and TNF also differed in their efficiency for RANTES generation with TNF being the more potent stimulant, IL-1 in comparison was a poor agonist. Similar findings on the specificity of RANTES regulation by IL-1 and TNF have been reported in human synovial fibroblasts (Rathanaswami *et al.* 1993), human umbilical vein endothelial cells (Marfaing-Koka *et al.* 1995) and a mouse MC cell line (Wolf *et al.* 1993).

The kinetics and specificity of RANTES induction in human MC is clearly different to that of IL-8 and MCP-1, which are expressed much earlier (maximal 4-8 hours) and

have a similar time course of expression over the first 8 hours following induction by either IL-1 or TNF. IL-1 was the more potent stimulant for IL-8 peptide production, while previous studies show IL-1 and TNF have similar efficacy for MCP-1 production in human MC, measured after 18 hours (Brown *et al.* 1991b; Brown *et al.* 1992). Relatively late induction of RANTES mRNA expression has been previously reported in mitogen and antigen stimulated peripheral blood T cells, which express RANTES 3 to 5 days after activation, in comparison to the rapid (4-6 hours) upregulation of other C-C chemokines (Nelson *et al.* 1993). Late induction of RANTES expression was also observed in human synovial fibroblasts and mouse MC, as mRNA levels peaked 24 hours after TNF stimulation (Rathanaswami *et al.* 1993; Wolf *et al.* 1993). Both these studies lend support to my observations.

Interestingly, a similar sustained time course for MCP-1 mRNA expression, and to a lesser extent for IL-8 mRNA expression was observed following TNF stimulation of MC, in comparison to IL-1. To my knowledge, this difference in the kinetics of IL-1 and TNF induced chemokine expression has not been previously reported in MC or other cell types. The results demonstrate that chemokine regulation by IL-1 and TNF in MC may differ not only in the potency of these stimuli, but also in the kinetics of production. Some differences must therefore exist between the signal transduction pathways employed by these two inflammatory cytokines, which is of interest as IL-1 and TNF are thought to induce similar post-receptor signal transduction pathways, as they share many biological responses (Le & Vilcek, 1987). The nature of the signalling pathways employed remains to be fully elucidated (O'Neill, 1995).

The observed difference in kinetics of chemokine induction in cultured human MC, with the early induction of IL-8 and MCP-1, the delayed induction of RANTES and the markedly sustained expression of TNF induced MCP-1 and RANTES, correlates well with the reported kinetics of leukocyte infiltration into inflamed glomeruli in rat models of glomerulonephritis (Tam *et al.* 1996; Stahl *et al.* 1993). Tam *et al* (1996) demonstrated a rapid, but transient infiltration of neutrophils and a sustained increase of monocytes in anti-GBM nephritis in rats, while a rapid and sustained influx of monocytes was observed in experimental mesangioproliferative GN (Stahl *et al.* 1993).

Induction of disease in these models resulted in enhanced glomerular expression of the chemokines MCP-1 and MIP-2, which correlated with the kinetics of leukocyte infiltration (Tam *et al.* 1996; Stahl *et al.* 1993). These findings support an important role for MC-derived chemokines in the progression of renal inflammation.

The protein synthesis inhibitor cycloheximide had disparate effects on IL-1 and TNF induced RANTES gene expression. CHX treatment resulted in superinduction of IL-1 induced RANTES mRNA levels, indicating secondary protein(s) were not required for the IL-1 response. Superinduction may result from inhibition of the synthesis of negative regulatory elements (nucleases, repressors) that are co-induced (Baggiolini *et al.* 1994). In marked contrast, CHX treatment inhibited TNF induced RANTES expression in MC, indicating the TNF induction of RANTES mRNA transcripts was protein synthesis dependent and thus secondary proteins may be involved. These observations are supported by similar findings in human synovial fibroblasts where TNF, but not IL-1 induced RANTES gene expression was inhibited by CHX (Rathanaswami *et al.* 1993). The identity of the secondary protein(s) involved in the TNF response in MC and their mechanism of action remains to be fully defined. However, my experiments to date demonstrate that endogenous production of IL-1 by TNF activated MC does not play a role in the TNF induction of RANTES, since pretreatment of MC with IL-1ra had no effect on the TNF response at concentrations which completely inhibited IL-1 stimulated RANTES production. Furthermore, results from the TNF neutralising antibody experiments indicate that the secondary protein(s) produced in TNF activated MC were unable to stimulate RANTES production in the absence of active TNF. The secondary protein(s) may therefore act to modulate the TNF response, causing upregulation of RANTES mRNA expression. The sustained time course of RANTES expression observed after TNF stimulation, in contrast to IL-1, lends to speculation that the secondary protein(s) may be involved at the level of mRNA stability. Interestingly, a recent report by A.Walz *et al.* demonstrated that CHX inhibited IL-1 induced ENA-78 mRNA expression, while superinducing IL-1 induced IL-8 expression in human monocytes (Walz *et al.* 1993). On analysing the time courses of ENA-78 and IL-8 mRNA expression, both chemokines were found to be induced initially with similar kinetics by IL-1. However, ENA-78 expression was more

sustained and remained elevated for over 72 hours, while IL-8 expression declined. Together these findings suggest a link between the involvement of secondary protein(s) in cytokine induced chemokine expression and a sustained time course of chemokine expression.

Preliminary studies show that CHX treatment resulted in superinduction of both IL-1 and TNF induced IL-8 mRNA expression in MC. CHX treatment also resulted in superinduction of IL-1 induced MCP-1 mRNA transcripts, while causing only a small potentiation of TNF induced MCP-1 mRNA levels. Superinduction of IL-1 and TNF induced IL-8 and MCP-1 expression has been previously reported in several human cell types, including endothelial cells (Dixit *et al.* 1990), smooth muscle cells (Colotta *et al.* 1992) and fibroblasts (Rathanaswami *et al.* 1993). These results indicate that secondary proteins are not involved in either IL-1 or TNF induced IL-8 or MCP-1 expression. These latter findings do not support my earlier suggestion that the role of secondary proteins in the TNF induction of RANTES mRNA expression may be at the level of mRNA stabilization, since secondary proteins were not involved in the TNF induction of IL-8 or MCP-1 mRNA transcripts in MC, yet a sustained time course of IL-8 and MCP-1 gene expression was observed following TNF stimulation. The role of secondary proteins in TNF induced RANTES expression therefore requires further investigation.

LPS has been shown to induce chemokine production in a variety of cell types, including human monocytes (Yoshimura *et al.* 1987), endothelial cells (Schroder & Christophers, 1989) and chondrocytes (Baggiolini *et al.* 1994). In the present study stimulation of MC with LPS failed to induce IL-8 or RANTES peptide production. An inability of LPS to stimulate IL-8 expression or production in human MC has been previously reported by Brown *et al* (1991b). In contrast, Kusner *et al* (1991) demonstrated significant IL-8 production by human MC after treatment with similar doses of LPS. Such discrepancies may be due to the use of different culture conditions, since only the latter study was performed in the presence of FCS, which is known to contain LPS-binding proteins that enhance LPS activity (Gallay *et al.* 1993).

Cultured human MC also express and secrete the mesangioproliferative cytokine IL-6 following IL-1 or TNF treatment, but not in response to LPS stimulation. In comparison to IL-1, TNF was a very poor stimulus for IL-6 production when experiments were performed on quiescent MC, which had been growth-arrested for 24 hours. Stimulation of the cells with TNF in the presence of 2% FCS however, caused a marked potentiation of the IL-6 response. Furthermore, serum itself induced IL-6 production, in the absence of any cytokine stimulation. The presence of a serum response element in the human IL-6 promoter may explain these findings (Ray *et al.* 1989). The latter work highlights the fact FCS contains a number of cytokines and growth factors (e.g. PDGF, bFGF) which may influence the cellular response during an experiment. The studies described in the subsequent chapters of this thesis were therefore carried out in the absence of serum, on growth-arrested MC in order to eliminate the effects of serum-derived factors. By performing the experiments in a more 'defined' system (i.e. in the absence of FCS or added growth factors) the ability of agents to modulate specifically IL-1 or TNF signalling mechanisms for IL-6, IL-8 or RANTES could be assessed, and conclusions about their signal transduction pathways drawn. MC were growth arrested in serum free medium for 24 hours to allow any effects of serum factors to diminish prior to the start of the experiment.

4. ROLE OF cAMP IN THE REGULATION OF IL-6, IL-8 AND RANTES PRODUCTION IN IL-1 OR TNF ACTIVATED HUMAN MESANGIAL CELLS

4.1. RATIONALE FOR STUDY

Little is known about the regulatory effects of cAMP on cytokine production in MC. This section of work was designed, therefore, to examine the role of cAMP in the regulation of IL-6 and IL-8 gene expression and peptide production in IL-1 stimulated human MC. During the course of this study, the ability of cAMP to modulate TNF induced IL-8 and RANTES peptide production was also assessed. The regulatory effects of cAMP on cytokine production were determined pharmacologically by utilising agents which elevate intracellular cAMP, including dibutyryl cAMP (db-cAMP), forskolin, 3-isobutyl-1-methyl-xanthine (IBMX) and cholera toxin (CT), an agent which elevates cAMP via ribosylation and activation of the stimulatory G protein, G_s.

4.2. RESULTS

4.2.1. Effect of cholera toxin or the purified B oligomer on cytokine induced IL-6 and IL-8 production by human mesangial cells

Pretreatment of MC with CT (10 - 250 ng/ml) for 5 hours prior to the addition of IL-1α resulted in a dose-dependent and significant ($p<0.05$) increase in IL-6 (Fig. 35a) and IL-8 (Fig. 35b) production, compared to cells treated with IL-1α alone. 250 ng/ml CT enhanced IL-6 and IL-8 production by 297 ± 56% and 106 ± 24% (mean ± SEM, n=5) respectively compared with the IL-1α control. Thus CT was 3 times more effective at stimulating IL-6 than IL-8 release from IL-1α activated MC.

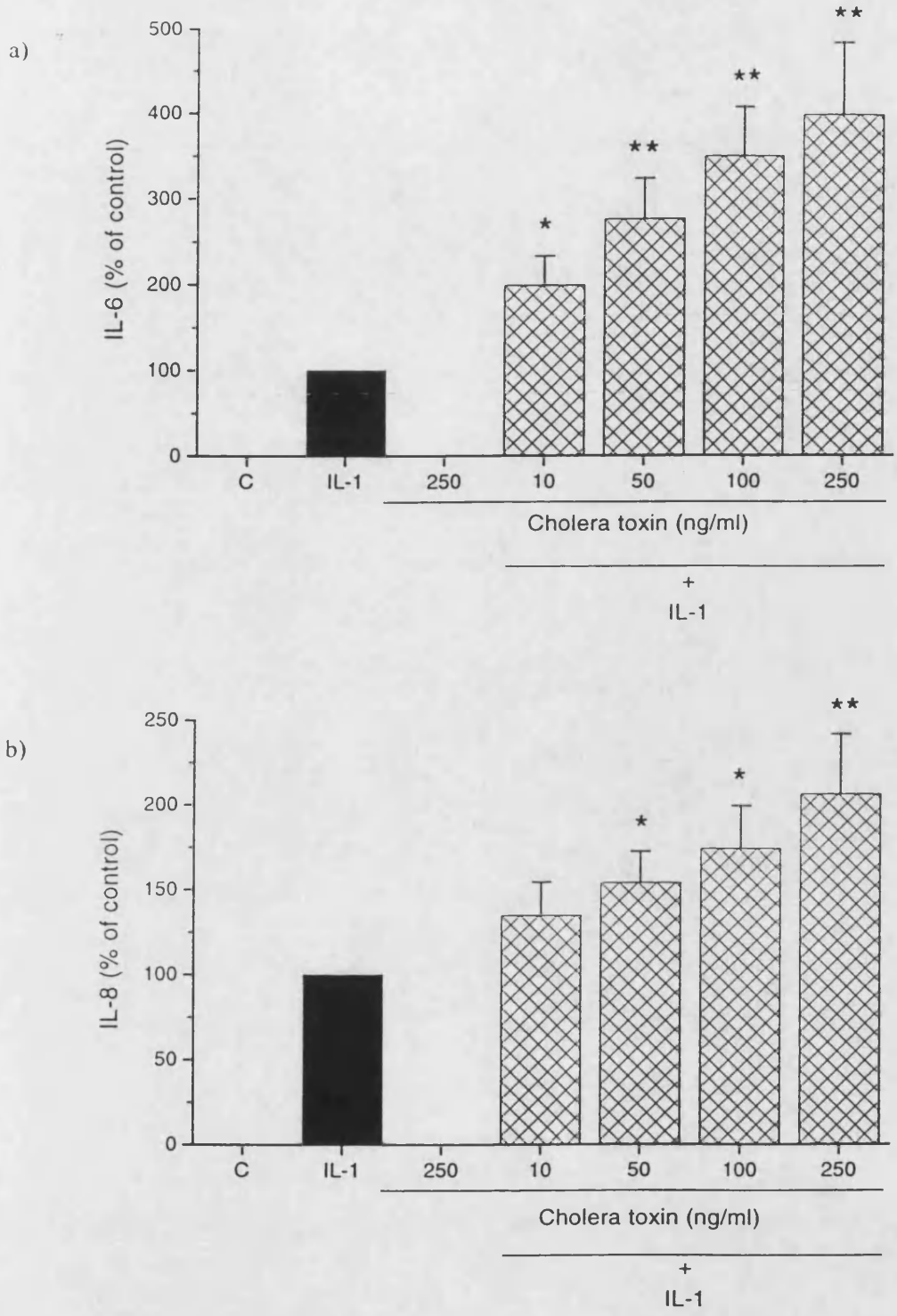


Figure 35. Cholera toxin potentiates IL-1 induced IL-6 and IL-8 peptide production by MC. MC cultured in 24 multi-well plates were pretreated with CT (10 - 250 ng/ml) for 5 hours prior to the addition of a sub-maximal dose of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Control wells (C) were treated with media alone for 23 hours. Cell supernatants were quantitated for extracellular IL-6 (a) and IL-8 (b) peptide by ELISA. Results are expressed as a % of the IL-1α control, where cells were stimulated with IL-1α alone for 18 hours, and are the mean ± SEM of n=5.

To assess whether the mechanism of CT potentiation was through activation of G_s by ADP-ribosylation and subsequent elevation of intracellular cAMP, the effects of the purified B oligomer of cholera toxin (CT-B) on IL-1α induced IL-6 and IL-8 peptide generation was investigated. CT-B can bind to the cell surface, but cannot modify G_s to activate the cAMP pathway due to the absence of S1 subunit, which is present in the holotoxin (CT). Surprisingly, pretreatment with CT-B resulted in enhanced IL-6 (Fig. 36a) and IL-8 (Fig. 36b) peptide production of 144 ± 27% and 88 ± 21% (mean ± SEM, n=4) respectively at 250 ng/ml, compared to the IL-1α control. The potentiation of IL-1α induced IL-6 production by CT-B was not dose-related across the concentration range employed (10 - 250 ng/ml), while CT-B had no significant effect on IL-1α induced IL-8 release between 10 and 100 ng/ml.

MC treated with CT or CT-B (250 ng/ml) alone for 23 hours showed no induction of IL-6 or IL-8 peptide above media control levels (Fig. 35 and 36).

4.2.2. Ability of cholera toxin and the purified B oligomer to ADP-ribosylate mesangial cell membrane proteins

ADP-ribosylation studies were performed to confirm CT was able to modify G_s proteins in the MC membrane within a 5 hour incubation with intact cells, and to check the CT-B preparation for contamination with S1 subunit. MC membranes treated with CT contained radiolabelled proteins of ~45 kDa and ~52 kDa (Fig. 37, lane 2). These correspond to the molecular weight of two known forms of G_s protein (Hepler & Gilman, 1992). To determine the time required for CT modification of the membrane pool of G_s, cells were treated with for 0, 0.5, 2 and 4 hours with CT (100 ng/ml). Membranes were then prepared and a second exposure of 15 minutes to CT in the presence of [^{32}P]-NAD was carried out. Figure 37 (lane 4) shows that after 2 hours contact of cells with CT all of the G_s protein was ADP-ribosylated. It was therefore concluded that the conditions in which the CT enhanced IL-6 and IL-8 production (5 hour preincubation) would have resulted in a near-total ADP-ribosylation and activation of G_s. In contrast, treatment of MC membranes with 50 μg/ml B oligomer instead of CT failed to generate the ~45 kDa and ~52 kDa bands (Fig. 37, lane 1) indicating no contamination of the CT-B preparation with S1 subunit.

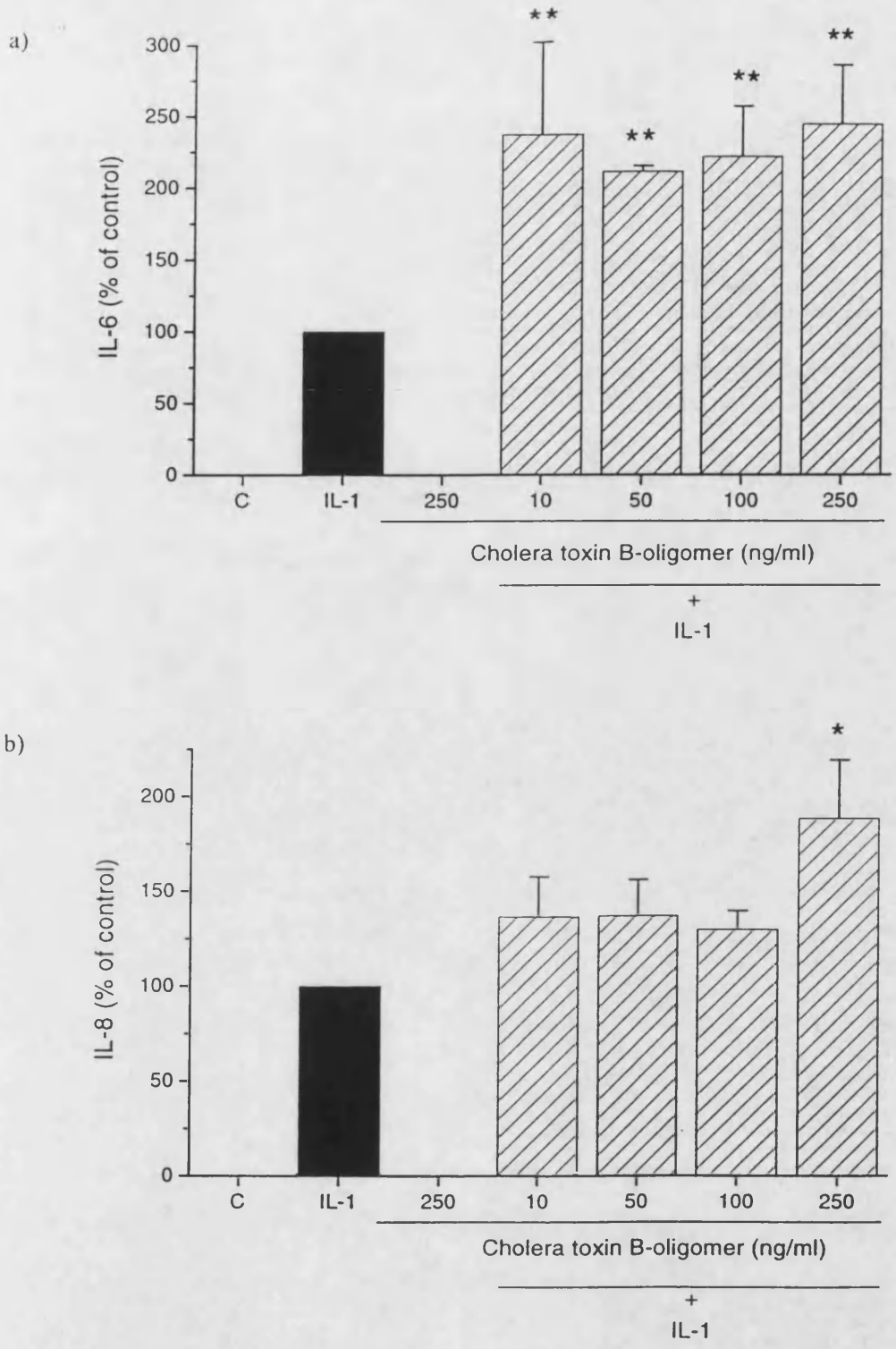


Figure 36. Effect of cholera toxin B oligomer on IL-1 induced IL-6 and IL-8 peptide production. MC were pretreated with CT-B (10 - 250ng/ml) for 5 hours prior to addition of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Cell supernatants were quantitated for extracellular IL-6 (a) and IL-8 (b) peptide by ELISA. Results are expressed as a % of the IL-1α control, where cells were stimulated with IL-1α alone for 18 hours, and are the mean ± SEM of n=4.

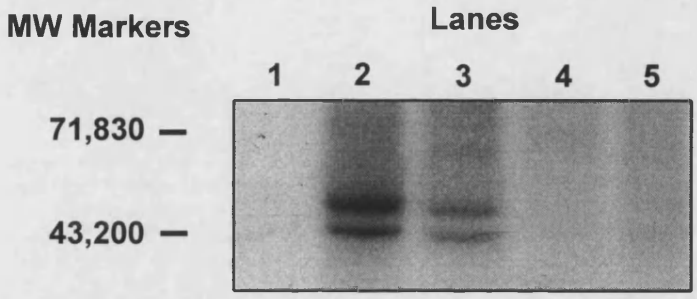


Figure 37. G proteins modified by cholera toxin or the B oligomer in MC membranes and time course of activation of G_s. MC cultured in 80 cm^2 flasks were pretreated with media alone (lanes 1, 2) or 100 ng/ml CT for 30 mins (lane 3), 2 hours (lane 4), or 4 hours (lane 5). Membranes were then extracted and 60 µg of membrane protein re-exposed to 50 µg/ml of pre-activated CT-B (lane 1) or CT (lanes 2-5) in the presence of [^{32}P]NAD for 15 mins at 30°C. Membrane proteins were resolved in a 12.5% SDS-polyacrylamide gel and autoradiographed. In lanes 3-5, the degree of G_s ribosylation that occurred on the initial exposure of intact cells to CT is inversely related to the amount of ribosylation observed on the autorad, which occurred during the second exposure of the cell membranes to CT in the presence of a [^{32}P]NAD label. The relevant part of the autorad is shown and is representative of 3 separate experiments carried out on MC isolated from 2 donor kidneys.

4.2.3. Ability of other cAMP-elevating agents to modulate cytokine induced IL-6 and IL-8 peptide production

The effect of agents that act via different mechanisms to elevate cAMP were investigated to clarify the role of cAMP in potentiating IL-1α induced IL-6 and IL-8 production. Db-cAMP is a membrane permeable, hydrolysis resistant cAMP analogue (Posternak *et al.* 1962), while forskolin activates the catalytic subunit of the adenylyl cyclase enzyme to cause increases in intracellular cAMP levels (Seaman & Daly, 1986). IBMX is a non-specific phosphodiesterase (PDE) inhibitor which prevents breakdown of cAMP within the cell (Nicholson *et al.* 1991).

Pretreatment of MC for 5 hours with forskolin (1 - 30 μM) or Db-cAMP (10 - 100 μM) resulted in a dose-dependent increase in IL-1α induced IL-6 peptide compared with IL-1α control (Fig. 38a). Maximal potentiation of IL-6 induced by either forskolin (10 μM) or by Db-cAMP (100 μM) was 125 ± 7% and 103 ± 10% (mean ± SEM, n=4) respectively compared to IL-1α alone. Similarly, a dose-dependent increase in IL-1α induced IL-6 was observed following a 1 hour IBMX pretreatment, 300 μM IBMX resulted in a 133 ± 43% (mean ± SEM, n=3) increase in IL-6 production (Fig. 38b). None of the cAMP-elevating agents employed enhanced IL-1α induced IL-6 production to the same extent as cholera toxin. In marked contrast, Db-cAMP, forskolin or IBMX pretreatment did not modify IL-1α induced IL-8 production (Fig. 39a and b).

The ability of cAMP to modify IL-1α induced serine esterase activity in PC60 cells was reported to be dependent upon the timing of cAMP elevation in relation to agonist stimulation, simultaneous addition of cAMP-elevating agents with IL-1α resulting in potentiation of IL-1 effects, while pretreatment with cAMP-elevating agents lead to inhibition (Schlegel-Haueter & Aebischer, 1990). The effect of timing of cAMP elevation upon IL-1α induced IL-6 and IL-8 production in MC was therefore investigated. Addition of Db-cAMP (30, 100 μM) simultaneously with IL-1α (Fig. 40a), or 5 hours after IL-1α stimulation (Fig. 40b) resulted in similar potentiation of IL-6 peptide production. Simultaneous addition of Db-cAMP with IL-1α had no significant effect on IL-8 production (Fig. 41).

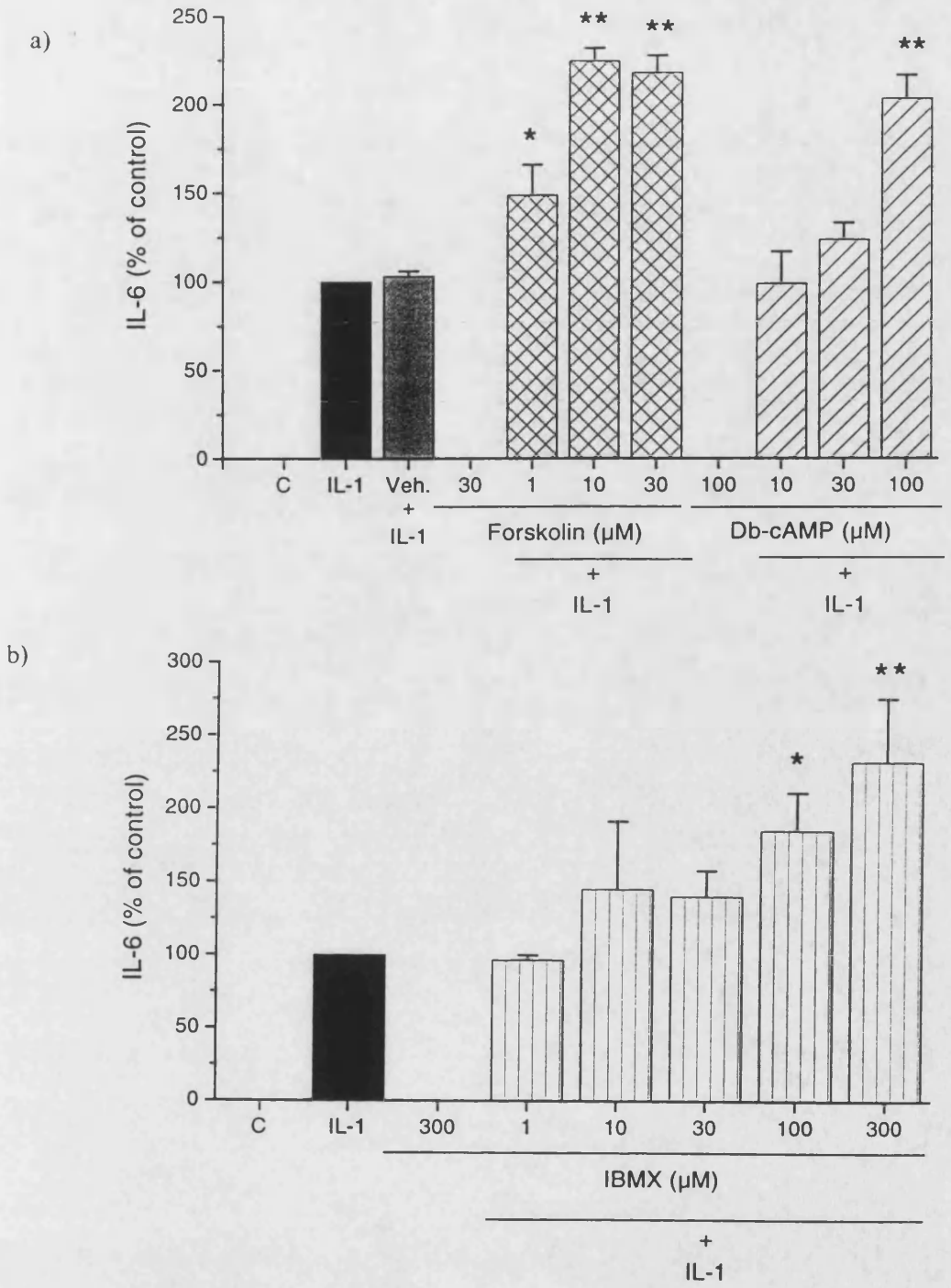


Figure 38. Effect of cAMP-elevating agents on IL-1 induced IL-6 peptide release by MC. Cells cultured in 24 multi-well plates were pretreated with either forskolin (1 - 30 μM), Db-cAMP (10 - 100 μM) (a) or IBMX (1 - 300 μM) (b) for 5 hours before addition of IL-1α (3 ng/ml) to the drug-containing media for 18 hours. The vehicle control (Veh) was 0.03% DMSO, which corresponds to the percentage DMSO present in the top concentration of forskolin used. Antigenic IL-6 levels in culture supernatants were quantitated by ELISA. Results are expressed as % of IL-1α controls and are the mean ± SEM of n=4 and n=3 respectively for the forskolin/Db-cAMP (a) and IBMX (b) data.

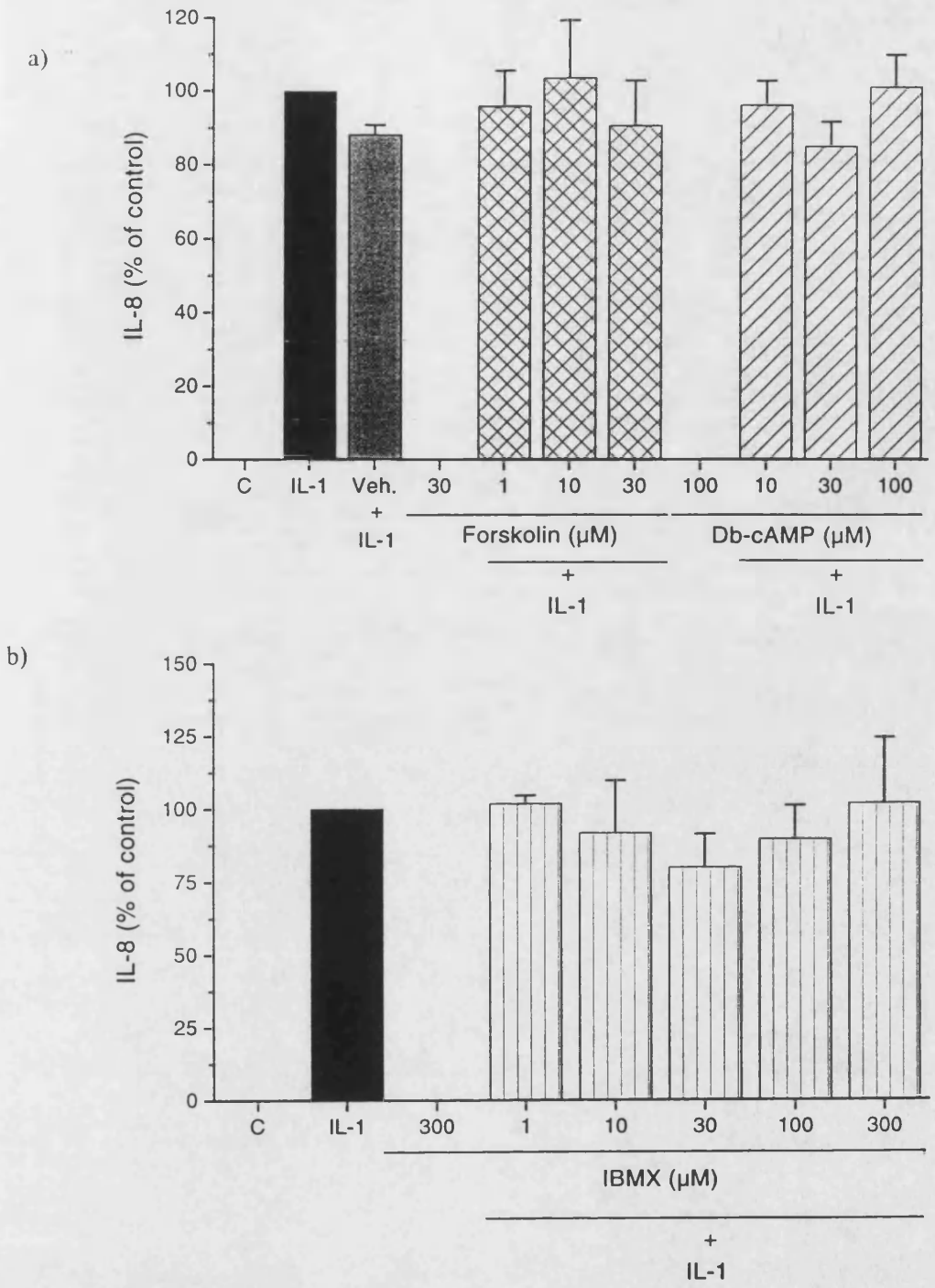
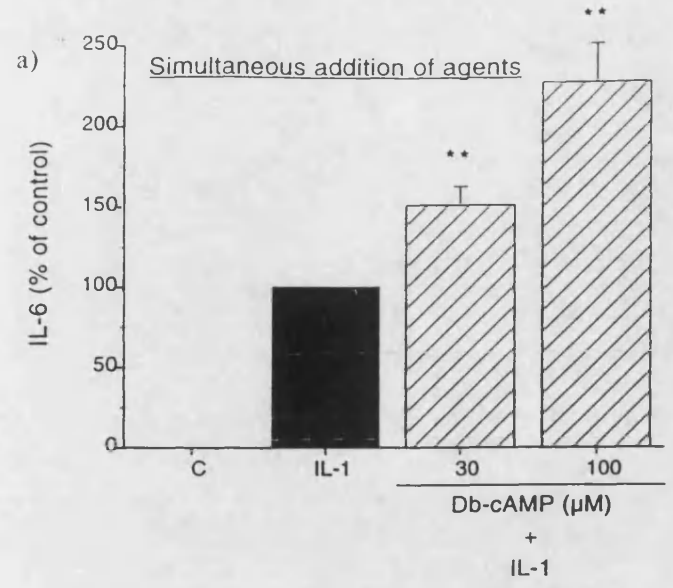


Figure 39. Effect of cAMP-elevating agents on IL-1 induced IL-8 peptide release by MC. Cells were pretreated with forskolin (1 - 30 μM), Db-cAMP (10 - 100 μM) (a) or IBMX (1 - 300 μM) (b) for 5 hours before addition of IL-1α (3 ng/ml) to the drug-containing media for 18 hours. The vehicle control (Veh) was 0.03% DMSO, which corresponds to the percentage DMSO present in the top concentration of forskolin used. Antigenic IL-8 levels in culture supernatants were quantitated by ELISA. Results are expressed as % of IL-1α controls and are the mean ± SEM of n=4 and n=3 respectively for the forskolin/Db-cAMP (a) and IBMX (b) data.



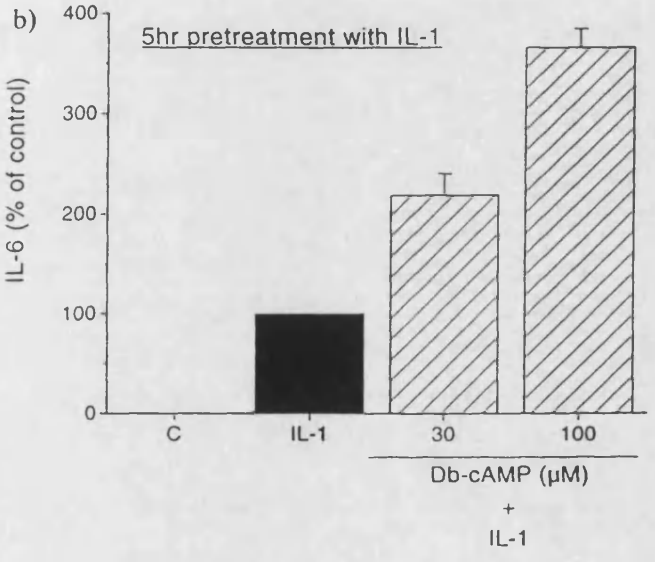


Figure 40. Db-cAMP upregulates IL-1 induced IL-6 peptide production independently of the timing of addition of the agents. Db-cAMP (30, 100 μM) was added to MC either simultaneously with IL-1α (3 ng/ml) for 18 hours (a), or 5 hours after IL-1α addition for a further 13 hours (b). Cell supernatants were quantitated for extracellular IL-6 peptide by ELISA. Result in fig. 40(a) and (b) are expressed as % of IL-1α control and are the mean ± SEM of n=7 and n=1 respectively.

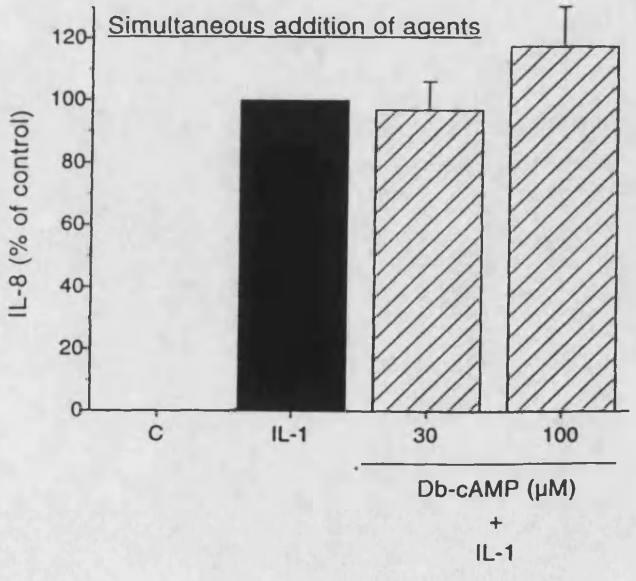


Figure 41. Effect of the simultaneous addition of Db-cAMP with IL-1 on IL-8 peptide production. MC were stimulated with Db-cAMP (30, 100 μM) and IL-1α (3 ng/ml) for 18 hours. Cell supernatants were quantitated for extracellular IL-8 peptide by ELISA. Results are expressed as % of IL-1α control and are the mean ± SEM of n=7.

Investigation of the effects of elevated intracellular cAMP on TNFα induced IL-8 production showed pretreatment with Db-cAMP (10 - 100 μM), forskolin (1 - 30 μM) or IBMX (1 - 300 μM) for 1 hour did not modify TNFα induced IL-8 generation (Fig. 42a and b). Simultaneous addition of Db-cAMP (30, 100 μM) with TNFα also had no effect on IL-8 production (Data not shown). In comparison to IL-8, preliminary findings indicate Db-cAMP (30 - 100 μM) and forskolin (1 - 30 μM) potentiate TNFα induced IL-6 production (Data not shown).

None of the agents, when added alone to the cells for the duration of the experiment caused any induction of IL-6 or IL-8 peptide above media control levels (Fig. 38 - 42).

4.2.4. Effect of a specific type IV phosphodiesterase inhibitor, RO 20-1724 on IL-1 induced IL-6 and IL-8 production

Five families of phosphodiesterases (PDE) are known to exist (I to V), each composed of several isoforms and having different tissue distributions. RO 20-1724 is a selective inhibitor of the type IV, cAMP-specific PDE, which represents more than 90% of the total cAMP phosphodiesterase activity in kidney tissue (Nicholson *et al.* 1991; Hoey & Houslay, 1990). To determine whether RO 20-1724 is an effective inhibitor of cAMP-specific PDE in MC, the effect of RO 20-1724 on IL-1α induced IL-6 and IL-8 production was assessed. Surprisingly, pretreatment of MC with RO 20-1724 (10 to 300 μM) for 1 hour had no effect on IL-1α induced IL-6 or IL-8 peptide production (Fig. 43a and b). These findings may be due to the existence of isoforms of type IV PDE which show a 5-15 fold difference in their sensitivity to this inhibitor (Nicholson *et al.* 1991; Hoey & Houslay, 1990), MC may possess the more resistant isoform(s).

4.2.5. Effect of agents that elevate cAMP on IL-1 induced IL-6 and IL-8 mRNA expression

Agents which elevate cAMP potentiated IL-1α induced IL-6 peptide production, but were without effect on IL-8 production in MC. To determine if the enhancement of IL-6 was occurring at the level of gene expression, the effect of these agents on IL-6 and IL-8 mRNA expression was assessed. Figure 44 (a and b) shows that pretreatment with Db-cAMP (100 μM), forskolin (30 μM), CT (100 ng/ml) or CT-B (100 ng/ml) for 5

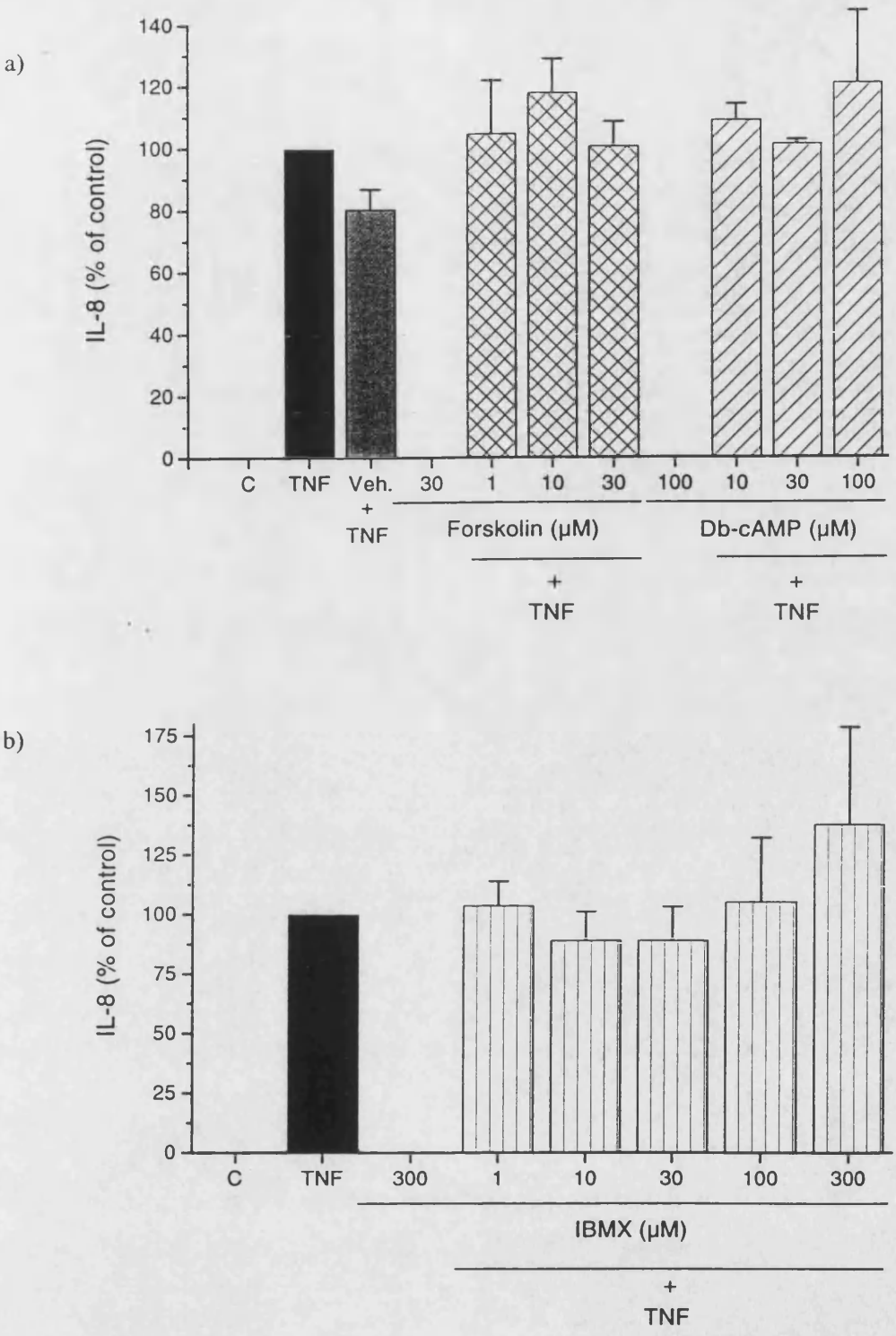


Figure 42. Effect of cAMP-elevating agents on TNF induced IL-8 peptide release. MC were pretreated with Db-cAMP (10 - 100 μM), forskolin (1 - 30 μM) (a) or IBMX (1 - 300 μM) (b) for 5 hours before addition of TNFα (300 ng/ml) to the drug-containing media for 18 hours. The vehicle control (Veh) was 0.03% DMSO, which corresponds to the percentage DMSO present in the top concentration of forskolin used. Cell supernatants were quantitated for extracellular IL-8 peptide by ELISA. Results are expressed as % of TNFα controls and are the mean ± SEM of n=4 and n=3 respectively for the forskolin/Db-cAMP (a) and IBMX (b) data.

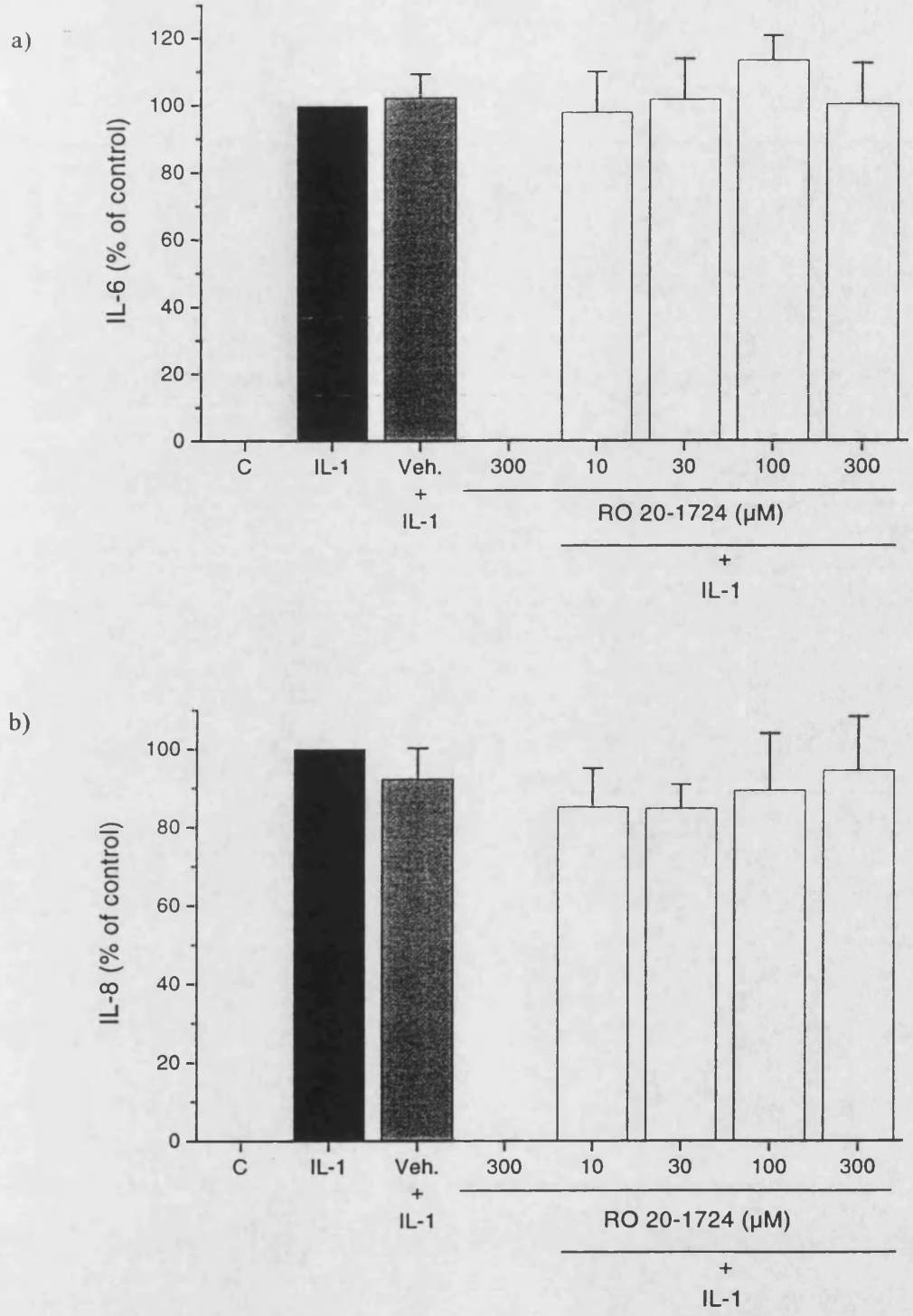


Figure 43. Effect of the type IV PDE inhibitor, RO 20-1724 on IL-1 induced IL-6 and IL-8 peptide production. MC were pretreated with RO 20-1724 (10 - 300 μM) for 1 hour prior to addition of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Cell supernatants were quantitated for extracellular IL-6 (a) and IL-8 (b) peptide by ELISA. Results are expressed as % of the IL-1α control and are the mean ± SEM of n=3.

hours, or IBMX (300 μM) for 1 hour enhanced IL-1α induced IL-6 mRNA levels, compared to cells treated with IL-1α alone.

In comparison, figure 45 demonstrates that pretreatment with IBMX (300 μM) or CT-B (100 ng/ml) had no effect on IL-1α induced IL-8 mRNA levels compared with the IL-1α control, while pretreatment with either Db-cAMP (100 μM), forskolin (30 μM) or CT (100 ng/ml) resulted in a small potentiation of IL-1α induced IL-8 mRNA transcripts.

None of the cAMP-elevating agents alone induced either IL-6 or IL-8 gene expression (Fig. 44 and 45).

4.2.6. Effect of Db-cAMP on IL-1 induced IL-6 mRNA stability

Messenger RNA degradation studies were carried out to determine if cAMP elevation was increasing IL-1α induced IL-6 expression by stabilization of IL-6 gene transcripts. MC were pretreated for 5 hours with media alone or Db-cAMP (100 μM) prior to the addition of IL-1α for 8 hours. Transcription was then arrested by the addition of actinomycin D (AcD) (5 μg/ml) and cultures were incubated for a further 0, 1, 2, 4, 8, 12 or 24 hours. Total cellular RNA was extracted at the end of each time point. Figure 46 shows IL-6 mRNA degradation in the presence of AcD was similar after stimulation of the cells with either IL-1α alone or Db-cAMP + IL-1α, indicating that the increase in IL-6 mRNA expression observed following treatment with agents that elevate cAMP was not due to an increase in IL-6 mRNA stability.

Addition of AcD for 24 hours resulted in a non-specific decrease in mRNA levels of the house-keeping gene, β-actin (Fig. 46).

4.2.7. Effect of Db-cAMP on IL-1 induced NF-κB activation in human mesangial cells

There is no constitutive activity of the transcription factor NF-κB in MC, however treatment with IL-1α results in marked NF-κB activation (Brown *et al.* 1993a). NF-κB binding sites have been located in the promoters regions of both the IL-6 and the IL-8

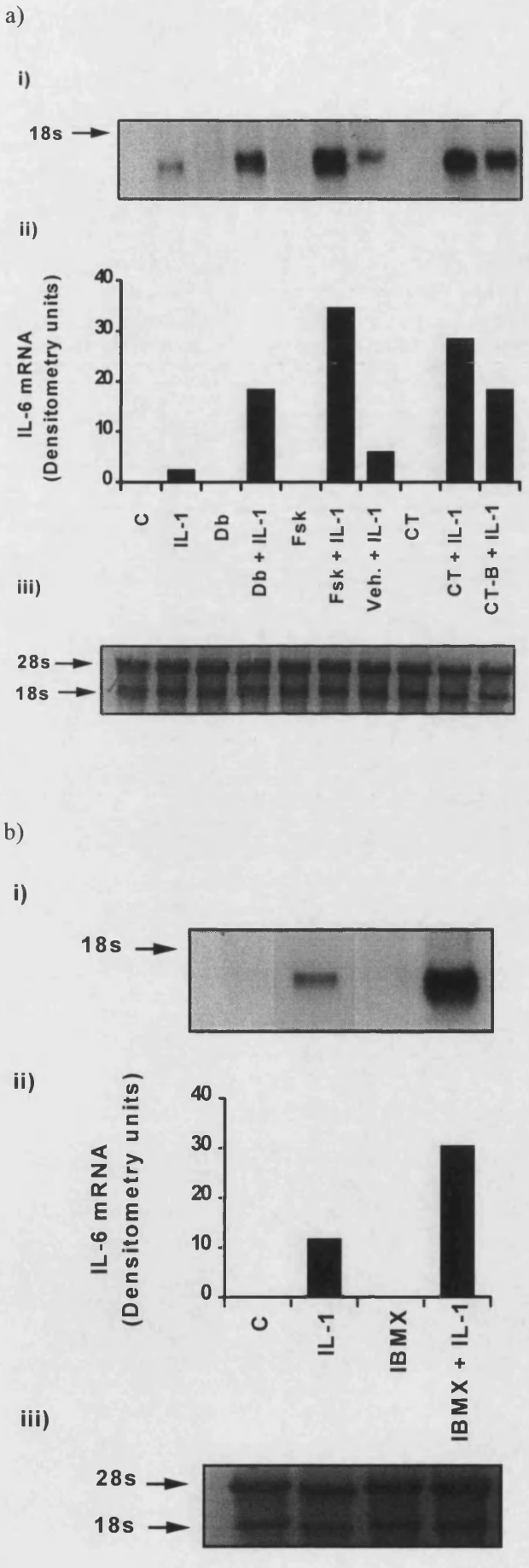


Figure 44. Effect of agents that elevate cAMP on IL-1 induced IL-6 mRNA expression. (a) MC cultured in 25 cm^2 flasks were pretreated for 5 hours with media alone, 100 μM Db-cAMP (Db), 30 μM forskolin (Fsk), 0.03% DMSO vehicle (Veh), 100 ng/ml CT or 100 ng/ml CT-B, before the addition of IL-1α (3 ng/ml) to the drug containing media. (b) MC were pretreated with media alone or 300 μM IBMX for 1 hour before addition of IL-1α. Control cells (C) were treated with media alone for the duration of the experiment.

After a 5 hour IL-1α stimulation, total cellular RNA was extracted and northern analysis carried out. Auto-radiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=4.

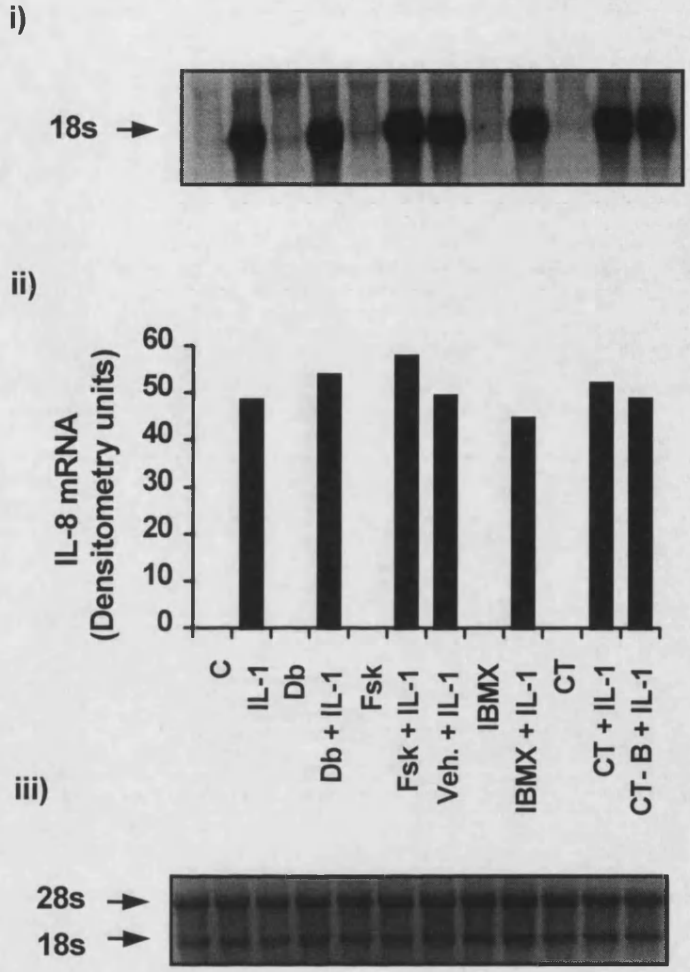


Figure 45. Effect of agents that elevate cAMP on IL-1 induced IL-8 mRNA expression. MC were pretreated for 5 hours with media alone, 100 μM Db-cAMP (Db), 30 μM forskolin (Fsk), 0.03% DMSO vehicle (Veh), 100 ng/ml CT, 100 ng/ml CT-B or for 1 hour with 300 μM IBMX. IL-1α (3 ng/ml) was then added to the drug containing media for 5 hours. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=4.

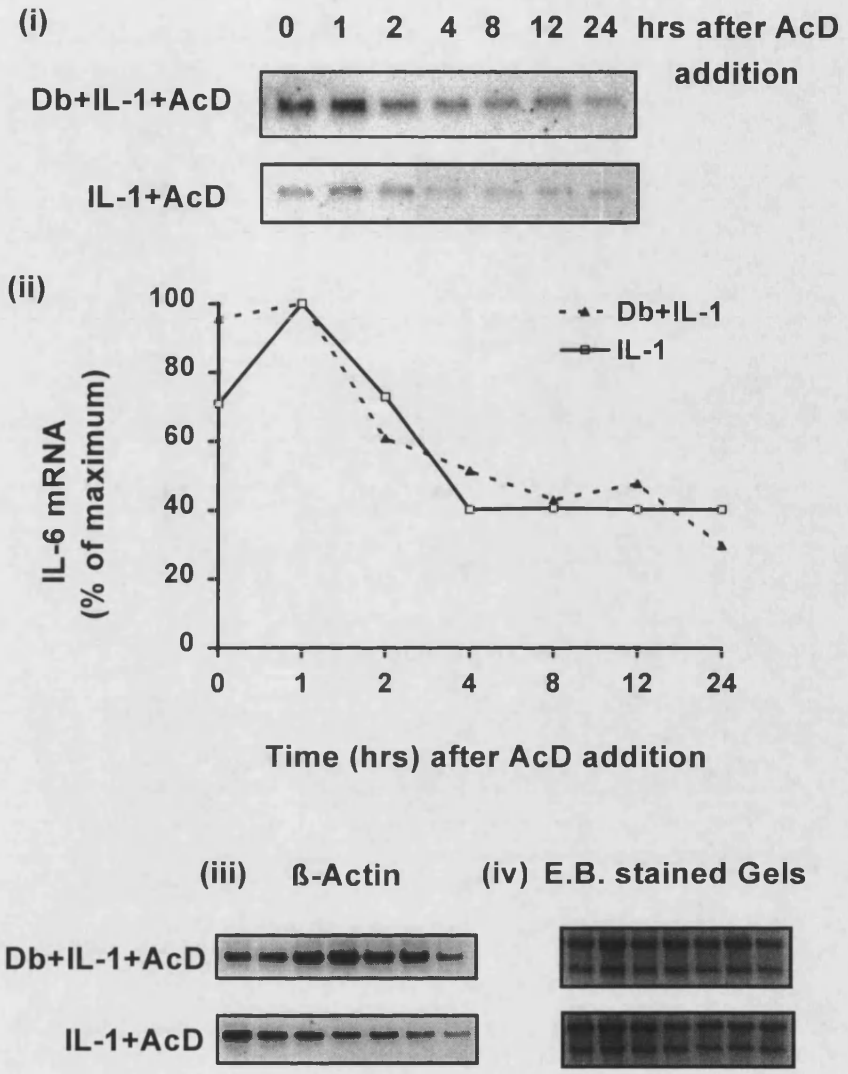


Figure 46. Effect of Db-cAMP on IL-1 induced IL-6 mRNA stability. MC culture in 25 cm^2 flasks were pretreated with media alone or 100 μM Db-cAMP for 5 hours prior to stimulation with IL-1α (3 ng/ml) for 8 hours. Actinomycin D (AcD) was then added to the drug containing media for 0, 1, 2, 4, 8, 12 or 24 hours. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs of IL-6 probed membranes (i) were analysed by densitometry and the results plotted using the absorbance units (ii). Identical membranes were probed for the house-keeping gene β-actin (iii) to assess equal loading, along with the ethidium bromide stained gels showing the 18S and 28S ribosomal RNA (iv). Representative blots are shown, and similar results were obtained in 2 other experiments.

genes, and NF-κB has been shown to play a role in their induction by IL-1α (Zhang *et al.* 1990; Shimizu *et al.* 1990; Mukaida *et al.* 1990). In addition, an involvement of NF-κB in mediating the cAMP activation of the IL-6 gene in a murine monocytic cell line has been demonstrated (Dendorfer *et al.* 1994). The effect of Db-cAMP on IL-1α induced NF-κB activation in MC was assessed to determine if the potentiation of IL-6 gene expression and production by cAMP was due to an increase in NF-κB activity. MC were pretreated with media alone or Db-cAMP (100 μM) for 5 hours prior to the addition of IL-1α for 0.5, 1, 2 or 6 hours, and nuclear extracts were prepared. Figure 47 shows pretreatment with Db-cAMP for 5 hours had no effect on NF-κB activation after stimulation with IL-1α for 0.5 or 6 hours, compared to the corresponding controls treated with IL-1α alone. Interestingly, Db-cAMP pretreatment resulted in partial inhibition of NF-κB activity after stimulation with IL-1α for 1 and 3 hours (Fig. 47). NF-κB activity was not observed in cells treated with media or Db-cAMP (100 μM) alone (data not shown). The results indicate that the potentiating effect of agents that elevate cAMP on IL-1α induced IL-6 mRNA expression is not occurring at the level of NF-κB activation.

4.2.8. Effect of indomethacin on IL-1 induced IL-6 and IL-8 production in mesangial cells

Cytokine activated human MC in culture are known to synthesize prostaglandins, in particular PGE_2 production is significantly increased above basal levels following IL-1α stimulation (Topley *et al.* 1989). MC respond to PGE_2 which upon receptor binding causes activation of G_s and elevation of intracellular cAMP (Friedlander *et al.* 1983). To investigate if endogenous PGE_2 production plays a role in the IL-1α induction of IL-6, MC were incubated with the cyclooxygenase inhibitor, indomethacin (0.1 - 10 μM) for 1 hour prior to stimulation with IL-1α. Figure 48 (a) shows that treatment with indomethacin resulted in almost complete inhibition of IL-1α induced PGE_2 production (IL-1α control = 473 ± 263 pg/ml PGE_2 after 18 hours (mean ± SEM, n=3)). Greater than 97% of PGE_2 production was inhibited at a concentration of 10 μM indomethacin, while a 37 ± 6% (mean ± SEM) reduction in IL-6 production was observed at the same concentration of indomethacin (Fig. 48b). In contrast, indomethacin treatment had no inhibitory effect on IL-1α induced IL-8 generation (Fig. 48c). The inhibitory effect of

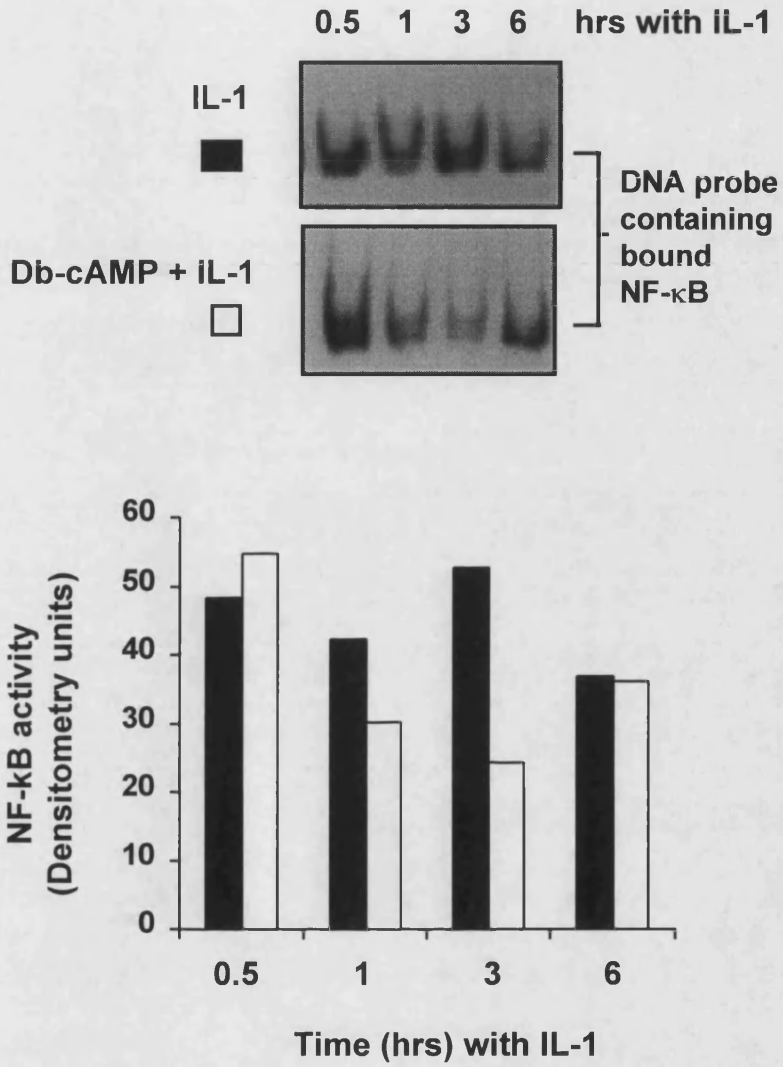


Figure 47. Effect of Db-cAMP on IL-1 induced NF-κB activation in MC. Cells culture in 25 cm^2 flasks were pretreated with media alone or 100 μM Db-cAMP for 5 hours prior to the addition of IL-1α for 0.5, 1, 2 or 6 hours. Nuclear extracts were prepared and gel shift assays performed. Autoradiographs were analysed by densitometry and the results plotted using the absorbance units. Representative blots are shown, n=2.

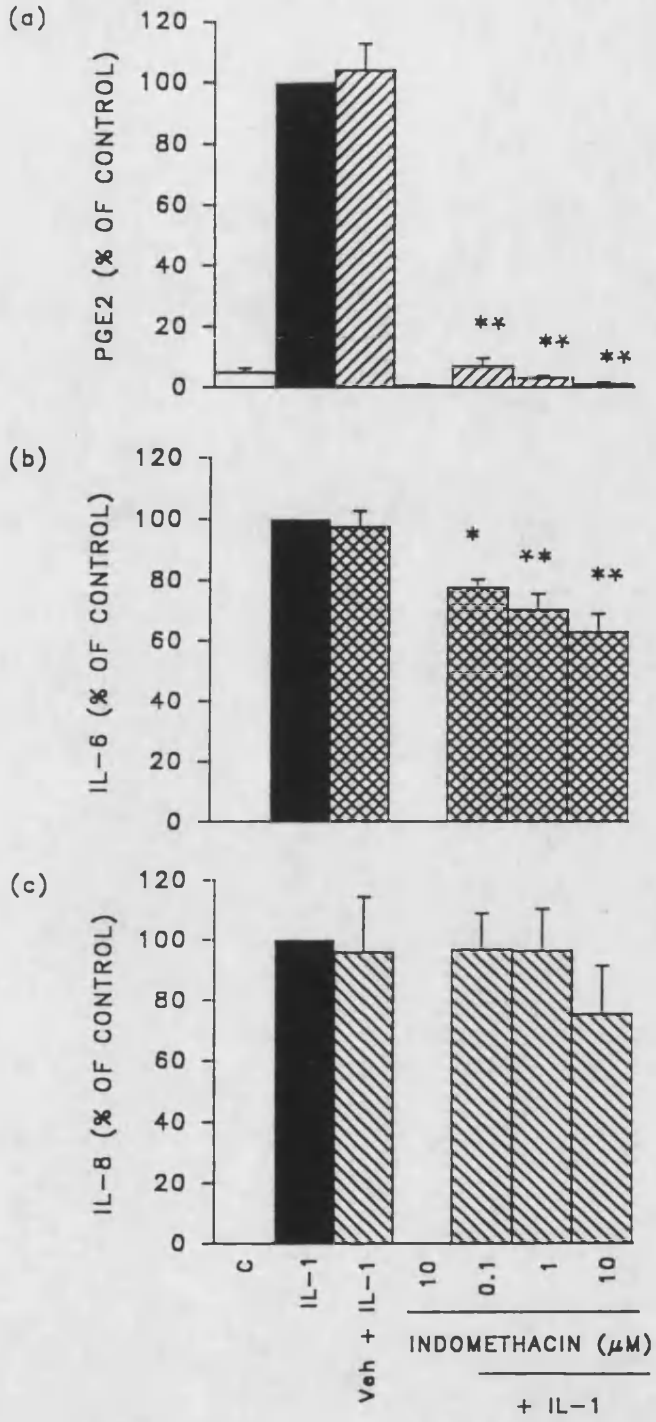


Figure 48. Effect of indomethacin on IL-1 stimulated PGE_2, IL-6 and IL-8 production by MC. Cells cultured in 24 multi-well plates were pretreated with indomethacin (0.1 - 10 μM) for 1 hour prior to stimulation with IL-1α (3 ng/ml) for 18 hours. The vehicle control (Veh) consisted of 0.036% ethanol, which corresponds to the percentage ethanol present in the top concentration of indomethacin used. The concentration of PGE_2 in cell supernatants was determined by RIA (a), while extracellular IL-6 (b) and IL-8 (c) peptide levels in cell supernatants were quantitated by ELISA. Results are expressed as % of the IL-1α control and are the mean ± SEM of n≥3.

indomethacin on PGE_2 and IL-6 production was not due to a toxic effect of the drug treatment, as cell viability as assessed by trypan blue exclusion was >90%. These results indicate that IL-6 production in MC following IL-1α stimulation may be potentiated by synthesis of PGE_2, probably acting via stimulation of adenylyl cyclase.

4.2.9. Regulatory effect of cAMP-elevating agents on RANTES production

The ability of cAMP to regulate RANTES peptide production in cytokine activated MC was investigated. Pretreatment of MC for 1 hour with either forskolin (1 - 30 μM) or Db-cAMP (10 - 100 μM) had no effect on IL-1α induced RANTES production (Fig. 49a). In contrast, both agents resulted in significant ($p<0.05$) inhibition of TNFα induced RANTES production, 57 ± 7% and 29 ± 6% (mean ± SEM, n=5) inhibition respectively was observed with 30 μM forskolin and 100 μM Db-cAMP (Fig. 49b).

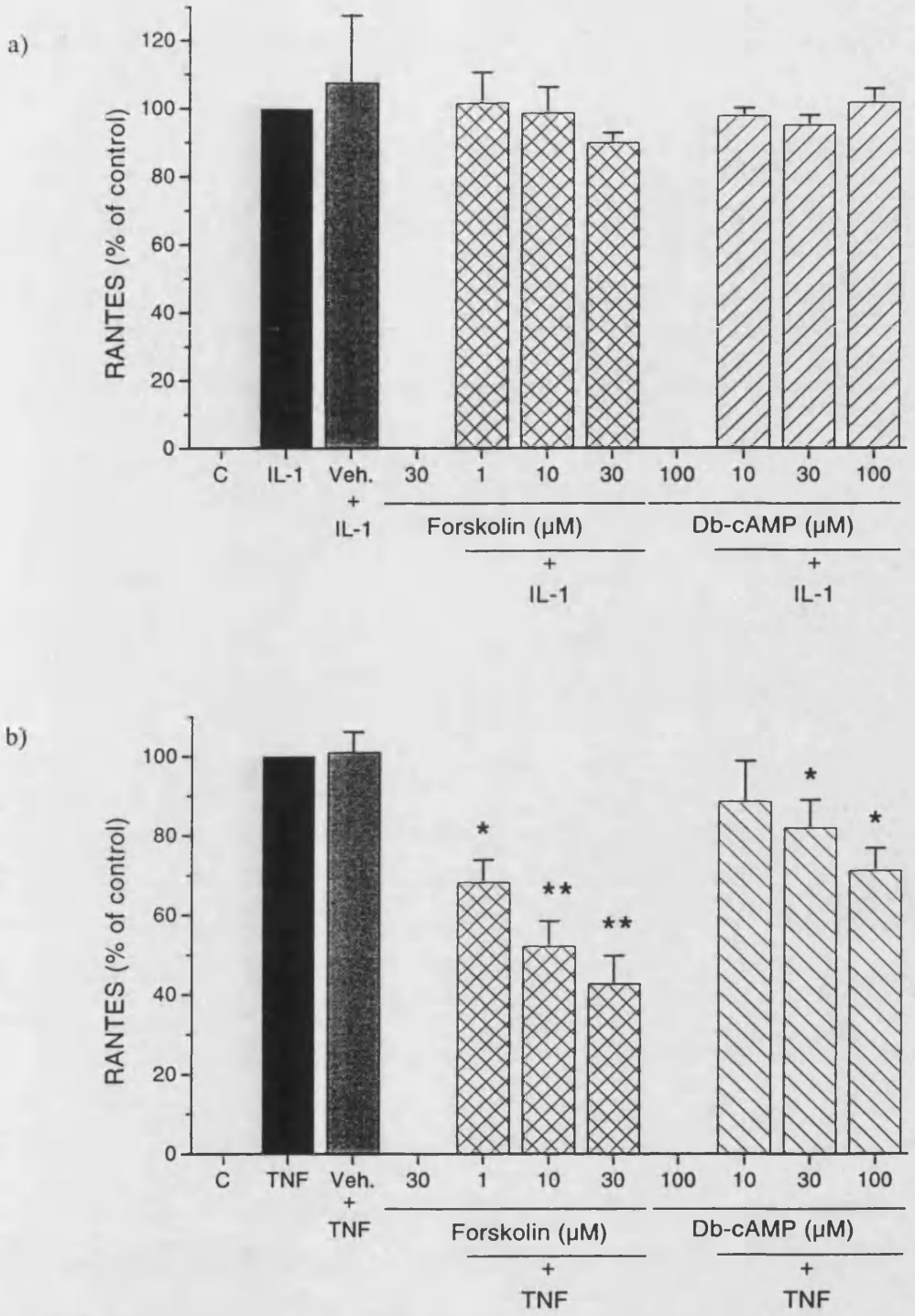


Figure 49. Effect of cAMP-elevating agents on IL-1 and TNF induced RANTES peptide production. MC were pretreated with forskolin (1 - 30 μM) or Db-cAMP (10 - 100 μM) for 1 hour prior to the addition of sub-maximal doses of IL-1α (1 ng/ml) (a) or TNFα (50 ng/ml) (b) to the drug containing media for 48 hours. The vehicle control (Veh) was 0.03% DMSO, which corresponds to the percentage DMSO present in the top concentration of forskolin used. Antigenic RANTES levels in culture supernatants were quantitated by ELISA. Results are expressed as % of the IL-1α (a) or TNFα (b) controls and are the mean ± SEM of n=5.

4.3. DISCUSSION

This area of work investigated the role of cAMP in the regulation of cytokine induced IL-6, IL-8 and RANTES production in MC. The results show that agents that elevate cAMP (db-cAMP, forskolin and IBMX) significantly potentiated expression of IL-6 gene transcripts and peptide production in response to IL-1, while having little or no effect on IL-8 gene expression and peptide production. In marked contrast, cAMP elevation markedly inhibited TNF stimulated RANTES production.

The bacterial toxin, cholera toxin (CT) was unique in that it was found to cause significant enhancement of both IL-6 and IL-8 peptide generation in response to IL-1. CT produced a marked enhancement of IL-6 transcripts, but caused only a minor, if any increase in IL-8 transcripts indicating that enhanced IL-8 peptide levels may result from post-transcriptional effects of CT. The data indicates that the effect observed with CT cannot solely be attributed to elevation of cAMP via G_s activation, since pretreatment of the cells with the purified B oligomer of CT (CT-B), which is devoid of ADP-ribosyltransferase activity, also resulted in significant potentiation of IL-1 induced IL-6 and IL-8 peptide production suggesting that cAMP-independent mechanisms may be involved. The flat dose-response of IL-6 upregulation by CT-B (50 - 500 ng/ml) may reflect saturation. The molecular weight of CT-B (~59 kDa) is less than 2 fold lower than the molecular weight of the holotoxin (~86 kDa) (Mekalanos, 1988) and thus similar amounts of CT-B are present in corresponding doses of the two agents. This implies the larger fold increase observed in IL-6 production following pretreatment with holotoxin compared to CT-B may be due to the presence of S1 subunit and consequently suggests some involvement of cAMP-dependent mechanisms in the CT upregulation of IL-6. In comparison, CT-B and holotoxin (at 250 ng/ml) induced similar fold increases in IL-8 generation suggesting cAMP independent mechanisms are involved. This latter suggestion is supported by the inability of cAMP-elevating agents which act via different mechanisms to CT, to potentiate IL-1 induced IL-8 production. CT-B is known to bind to a specific cell surface receptor, the sialic acid-containing glycosphingolipid, GM1. An ability of the CT-B / GM1 pathway to modulate cell growth, differentiation or T cell IL-2 expression via cAMP-independent mechanisms has been previously described, although the signalling

pathways involved remain unclear (Bremer *et al.* 1986; Lycke, 1993; Gouy *et al.* 1994; Woogen *et al.* 1993). Interestingly, the binding of CT-B to GM1 has been reported to increase intracellular calcium levels in neurones (Milani & Minozzi, 1992), the human Jurkat T cell line (Gouy *et al.* 1994), Swiss 3T3 fibroblasts (Spiegel & Panagiotopoulos, 1988) and rat thymocytes (Jeffrey Dixon *et al.* 1987). The Ca^{2+} response in Jurkat cells was shown to involve both an influx of extracellular Ca^{2+} and the release of Ca^{2+} from inositol 1,4,5-triphosphate-sensitive intracellular stores, suggesting the activation of plasma membrane Ca^{2+} channels and phospholipase C respectively by CT-B (Gouy *et al.* 1994). However, the induction of detectable Ca^{2+} responses in all these studies required the use of μg/ml doses of CT-B or the use of anti-CT-B crosslinking antibodies following pretreatment with ≥250 ng/ml CT-B (Milani & Minozzi, 1992; Gouy *et al.* 1994; Spiegel & Panagiotopoulos, 1988; Jeffrey Dixon *et al.* 1987). The contrasting dose-response curves for the CT-B upregulation of IL-1 induced IL-8 and IL-6 production in MC indicates Ca^{2+} responses may not be involved. Other pathways have been implicated in mediating CT-B actions. Recent findings have shown various gangliosides, including GM1 can modulate *in vitro* calmodulin-dependent enzyme activity (Higashi & Yamagata, 1992), and GM1, when bound and cross-linked by CT-B may interact with the cytoskeleton (Kellie *et al.* 1983). In addition, GM1 may mediate effects by interacting with integral membrane proteins and modulating their activity, inhibition of PDGF and EGF induced receptor phosphorylation by GM1 has been shown (Bremer *et al.* 1986). Studies have also indicated that CT may interfere with signalling pathways other than the cAMP pathway, an ability which is dependent on the ADP-ribosylating activity of the toxin (Imboden *et al.* 1986). Such action by CT is thought to account for expression of JE, the mouse monocyte chemokine in 3T3 cells (Qureshi *et al.* 1991). Thus the ability of CT to modulate a number of different signalling pathways through properties of both the S1 subunit and B oligomer may account for its potentiating effects on IL-1 induced IL-8 generation and explain the large increases seen in IL-6 generation, which could not be mimicked fully by the other cAMP agonists.

Studies in human FS-4 fibroblasts have shown an induction of IL-6 mRNA expression on stimulation with cAMP-elevating agents alone (Zhang *et al.* 1988a). Furthermore, cAMP was found to activate IL-6 transcription by a reporter gene assay in HeLa cells (Ray *et al.*

1988) and the murine monocytic cell line PU5-1.8 (Dendorfer *et al.* 1994). This contrasts with the present findings in human MC. The inability of cAMP-elevating agents alone to cause any induction of IL-6 mRNA or peptide could be linked to the fact the experiments were undertaken on quiescent MC, growth-arrested in serum-free medium for 24 hours before use. Serum itself is known to induce IL-6 expression (Ray *et al.* 1988) and the presence of serum immediately prior to agonist stimulation can result in pronounced increases in cAMP accumulation (Kreps *et al.* 1993). However, other studies undertaken on human lung fibroblasts which were not growth-arrested (Zitnik *et al.* 1993) or on human astrocytoma cells which were stimulated in the presence of low serum concentrations (Kasahara *et al.* 1990) also describe an inability of cAMP-elevating agents to stimulate IL-6 expression. This indicates that cell specific differences exist in IL-6 gene regulation, which cannot be explained solely by differences in culture conditions.

The ability of cAMP to enhance IL-6 generation in the presence of cytokines has been previously reported in a human astrocytoma cell line (Kasahara *et al.* 1990) and rat intestinal epithelial cell line (McGee *et al.* 1993). In contrast Zitnik *et al* (Zitnik *et al.* 1993) have shown cAMP to significantly inhibit IL-1 induced IL-6 mRNA and peptide production in a human lung fibroblast cell line. Furthermore, cAMP-elevating agents have been shown to down-regulate endotoxin induced IL-6 in Kupffer cells (Goss *et al.* 1993) and to have no effect on IL-6 generation in endotoxin stimulated mononuclear cells (Waage *et al.* 1990). The reason for the variations in the ability of cAMP to modulate IL-6 production is not clear, and is likely to involve cell specific differences. In addition, these findings may reflect an ability of factors such as the timing of cAMP elevation in relation to agonist stimulation (Schlegel-Haueter & Aebischer, 1990), the dose of cAMP-elevating agent employed (Renz *et al.* 1988), the agonist used (Hurme, 1990) and the activation state of the cell (Renz *et al.* 1988) to affect the type of regulation observed. In the present study, addition of db-cAMP simultaneously with IL-1, or 5 hours after IL-1 stimulation resulted in a similar potentiation of IL-1 induced IL-6 peptide production, as described with a 5 hour pretreatment of MC.

In vivo, prostaglandins are one of the main mediators known to exert modulatory effects on inflammatory events via activation of the cAMP pathway. Increased PGE_2 synthesis in

response to IL-1 in cultured MC has been shown previously (Topley *et al.* 1989). The present work extends these findings by demonstrating inhibition of IL-1 induced PGE_2 generation with indomethacin leads to a significant inhibition of IL-6 production, but is without effect on the IL-8 production. This suggests that endogenous PGE_2 generation in IL-1 stimulated MC operates via a positive feedback loop to upregulate IL-6 production, probably through elevation of intracellular cAMP. This hypothesis is supported by the lack of effect of indomethacin on IL-1 induced IL-8 generation, further supporting the data that IL-8 production in MC is not modulated by cAMP-elevating agents such as PGE_2.

From the results of this study, certain insights into the IL-1 signalling mechanisms for IL-6 and IL-8 can be drawn. In MC, the lack of induction of either IL-6 or IL-8 gene expression or production in the presence of cAMP-elevating agents alone indicates an inability of cAMP to mimic the actions of IL-1. The potentiating effect of cAMP on IL-6 production by IL-1 however does indicate that the IL-1 signalling pathway for IL-6 and the cAMP pathway interact. The ability of agents that elevate cAMP to potentiate IL-6 mRNA gene transcripts, as well as peptide production indicates that this cross-talk between the pathways is occurring at the level of gene expression. mRNA degradation studies showed the time course of IL-1 induced IL-6 mRNA degradation was the same in both the presence or absence of db-cAMP, indicating cAMP elevation was not increasing IL-6 expression by stabilization of IL-6 mRNA transcripts. The potentiating effect of cAMP on IL-1 induced IL-6 expression may therefore be occurring at the level of transcription. Nuclear run-on assays would need to be performed to confirm this. Previous studies have indicated the transcription factor NF-κB may play an important role in mediating IL-1 induction of IL-6 gene expression (Zhang *et al.* 1990; Shimizu *et al.* 1990), while reporter gene assays performed in the murine monocytic cell line PU5-1.8 demonstrated an involvement of NF-κB in the cAMP activation of the IL-6 gene (Dendorfer *et al.* 1994). In these studies mutations in the NF-κB binding site of the IL-6 promoter, which impaired the binding of NF-κB, markedly reduced the induction of IL-6 by IL-1 or cAMP-elevating agents. Preliminary experiments in MC however indicate the mechanism of cAMP potentiation of IL-1 induced IL-6 expression is not at the level of NF-κB activation, as db-cAMP did not upregulate IL-1 induced NF-κB activity. Furthermore, treatment of MC with db-cAMP alone caused no detectable induction of NF-

κB activity. Other transcription factors, such as NF-IL-6, which are implicated in both the IL-1 and cAMP induction of IL-6 gene expression may be involved (Dendorfer *et al.* 1994; Akira *et al.* 1990b).

The inability of cAMP to modulate IL-1 stimulated IL-8 generation highlights differences between the regulation of IL-6 and IL-8 in human MC. In addition, the lack of effect of cAMP on IL-8 production in response to TNF indicates both the IL-1 and TNF signalling pathway(s) for IL-8 are independent of the cAMP pathway. An inability of cAMP to regulate IL-8 production has been previously demonstrated in LPS stimulated neutrophils (Wertheim *et al.* 1993). In contrast, cAMP was found to inhibit IL-8 production in LPS stimulated monocytes (Standiford *et al.* 1992). These findings suggest the cAMP regulation of IL-8 production may be both cell and stimulus specific.

In the present study, the regulatory effects of cAMP on RANTES production in MC was also investigated. Treatment of MC with either forskolin or db-cAMP significantly inhibited RANTES peptide production ($p<0.01$) in response to TNF, but had no effect on the IL-1 induced response for this chemokine. The poor induction of RANTES by IL-1 may account for the inability of cAMP to modulate this response in MC, alternatively differences between the IL-1 and TNF signalling pathways for RANTES generation may be involved. The regulatory effects of cAMP on TNF induced RANTES production are in agreement with the recent observations of Satriano *et al* (1993) who found that increasing cAMP suppressed TNF induced MCP-1 expression at the transcriptional level in mouse MC. Similar results have also been reported by Rovin and Tan (1994) for MCP-1 in human MC, except in this case it was the IL-1 induced response which was attenuated. More recently both groups have demonstrated that raising intracellular cAMP reduced IL-1 or TNF stimulated NF-κB activation in MC as well as decreasing MCP-1 mRNA expression (Rovin *et al.* 1994a; Satriano & Schlondorff, 1994b). As a NF-κB binding site has been identified in the 5'-promoter region of the MCP-1 gene (Shyy *et al.* 1990), these results suggest that NF-κB may play a role in the IL-1 and TNF induction of MCP-1, and furthermore, NF-κB may be the molecular target for the inhibitory action of cAMP on MCP-1 expression. This latter suggestion is supported by a recent study in T lymphocytes which showed that mutation

of the NF-κB binding site in the promoter region of the IL-2 gene, that prevented NF-κB binding, resulted in the loss of cAMP-mediated suppression of IL-2 induction (Neumann *et al.* 1995). An NF-κB binding site has also been identified in the 5'-promoter region of the RANTES gene (Nelson *et al.* 1993), lending to speculation that the mechanism of cAMP regulation of TNF induced RANTES production in human MC may occur through inhibition of NF-κB activity. However, modification of NF-κB activity by cAMP is unlikely to account fully for the regulatory effects of cAMP on chemokine production since studies have shown NF-κB was essential for IL-1 or TNF induced IL-8 expression in more than 6 different cell lines examined (Mukaida *et al.* 1994), while IL-8 production was unaffected by elevation of intracellular cAMP in MC. Further studies are therefore necessary to determine the mechanism of cAMP regulation and to identify the factor(s) required to confer cytokine and chemokine responsiveness to cAMP regulation.

5. INVESTIGATION OF THE ROLE OF PERTUSSIS TOXIN-SENSITIVE G PROTEINS IN IL-1 SIGNALLING FOR IL-8 AND IL-6 IN HUMAN MESANGIAL CELLS

5.1. RATIONALE FOR STUDY

The molecular mechanisms by which IL-1 activates cells are not clear and remain one of the major challenges of immunology (O'Neill, 1995). A major amplification in signal is thought to occur following IL-1 binding to its' receptor as receptor occupancy studies have indicated less than 10 IL-1 receptors per cell need to be activated in order to elicit a biological response (Stylianou *et al.* 1992; Curtis *et al.* 1989). Amplification of hormonal signalling is classically known to occur through coupling of receptors to heterotrimeric G proteins, with the subsequent generation of second messengers. Several groups have published evidence for a G protein involvement in both IL-1 and TNF signalling. Much of this is based upon the inhibitory effects of the bacterial pertussis toxin (PT) on post-receptor events triggered by either IL-1 or TNF (Dobson *et al.* 1989; Chedid *et al.* 1989; O'Neill *et al.* 1990a; Rollins *et al.* 1991; Imamura *et al.* 1988; Brett *et al.* 1989; Clark *et al.* 1988). The aim of this section of work was to investigate the role of PT-sensitive G proteins in the IL-1 signalling for IL-8 and IL-6 in human MC.

5.2. RESULTS

5.2.1. Ability of pertussis toxin or the purified B oligomer to modulate IL-1 induced IL-8 and IL-6 production in human mesangial cells

The effect of pertussis toxin on IL-1α induced IL-8 and IL-6 peptide production was assessed to determine whether PT-sensitive G proteins play a role in IL-1α signalling events in human MC. Pretreatment of MC with 50 to 500 ng/ml of PT for 5 hours prior

to the addition of IL-1α resulted in significant ($p<0.05$) inhibition of IL-8 (Fig. 50a) and IL-6 production (Fig. 50b), compared to cells treated with IL-1α alone. A 40 ± 11% (mean ± SEM, n=5) and 27 ± 4% (mean ± SEM, n=5) decrease in IL-1α induced IL-8 and IL-6 production respectively was observed after pretreatment with 50 ng/ml PT. The inhibition observed following PT treatment was not dose-dependent in the concentration range of 50 to 500 ng/ml, PT at the higher dose of 1000 ng/ml had no significant effect on IL-8 or IL-6 production.

To assess whether the mechanism of PT inhibition was through ADP-ribosylation of PT-sensitive G proteins and subsequent inhibition of G protein activity, the effects of the purified B oligomer of pertussis toxin (PT-B) on IL-1α induced IL-8 and IL-6 peptide production was investigated. PT-B can bind to the cell surface, but cannot modify G proteins due to the absence of S1 subunit which is present in the holotoxin (PT). Surprisingly, pretreatment with PT-B resulted in similar inhibition of IL-1α induced IL-8 production to that observed with the holotoxin, a 39 ± 10% (mean ± SEM, n=5) decrease in IL-1α induced IL-8 production was observed with 50 ng/ml PT-B (Fig. 51a). The inhibition was not dose-related in the concentration range of 50 to 500 ng/ml and at a 1000 ng/ml PT-B had no significant effect. Inhibition of IL-1α induced IL-6 production was also observed following PT-B treatment, at 50 ng/ml PT-B decreased IL-6 production by 18 ± 3% (mean + SEM, n=5) (Fig. 51b). The effect of PT-B on IL-6 production was significant ($p<0.01$) using ANOVA analysis, but did not reach statistical significance when further assessed using Dunnetts t-test. The inhibitory effects of PT and PT-B on IL-8 and IL-6 production were not due to a toxic effect of drug treatment, as cell viability assessed by trypan blue exclusion was >90%. Furthermore PT and PT-B did not interfere with either IL-8 or IL-6 antigenic detection by ELISA as addition of 100 ng/ml PT or PT-B to known standards of IL-8 and IL-6 had no effect on the ELISA results (data not shown).

Treatment of MC with PT or PT-B (500 ng/ml) alone for the duration of the experiment had no effect on basal IL-8 or IL-6 production (Fig. 50 and 51).

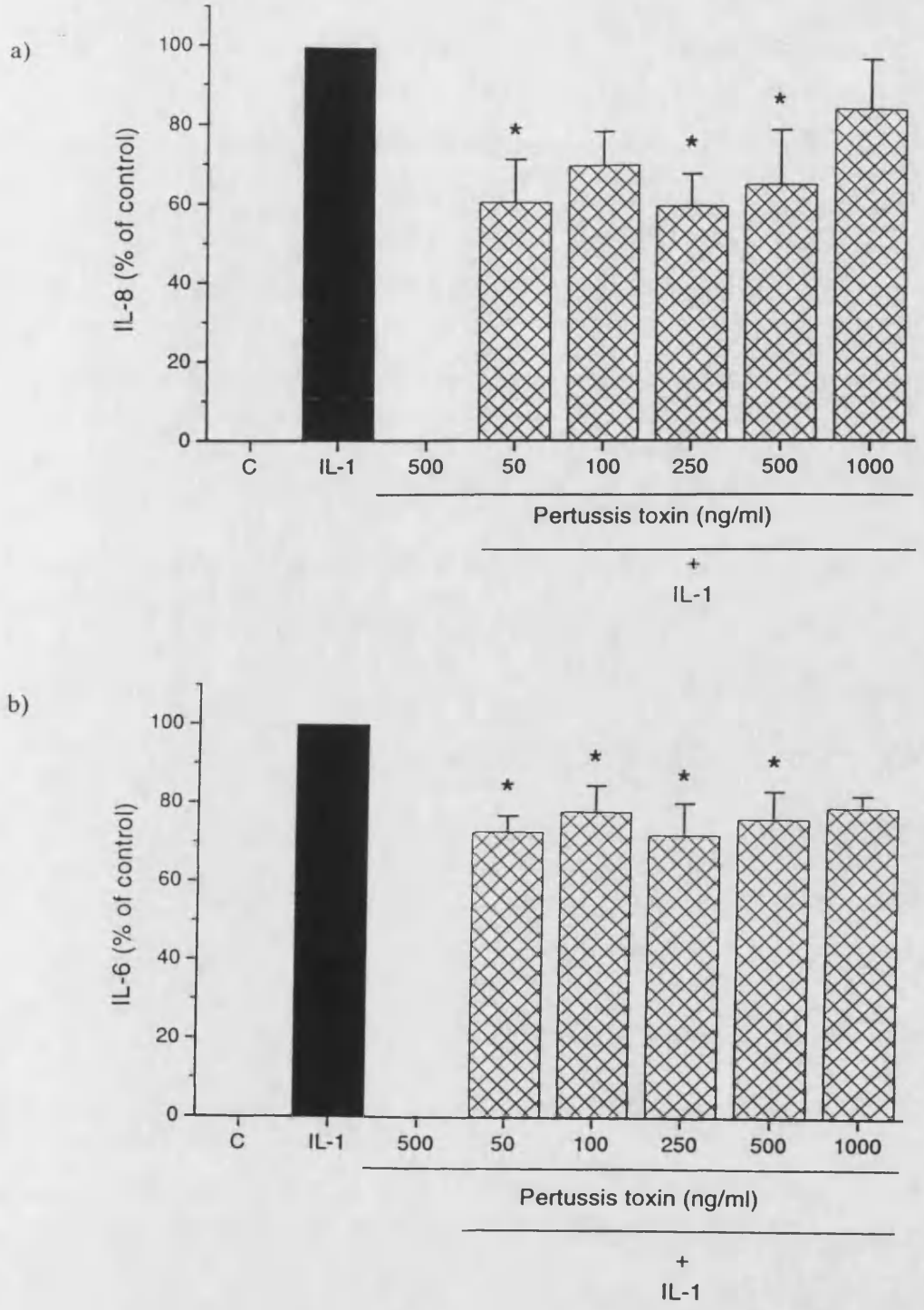
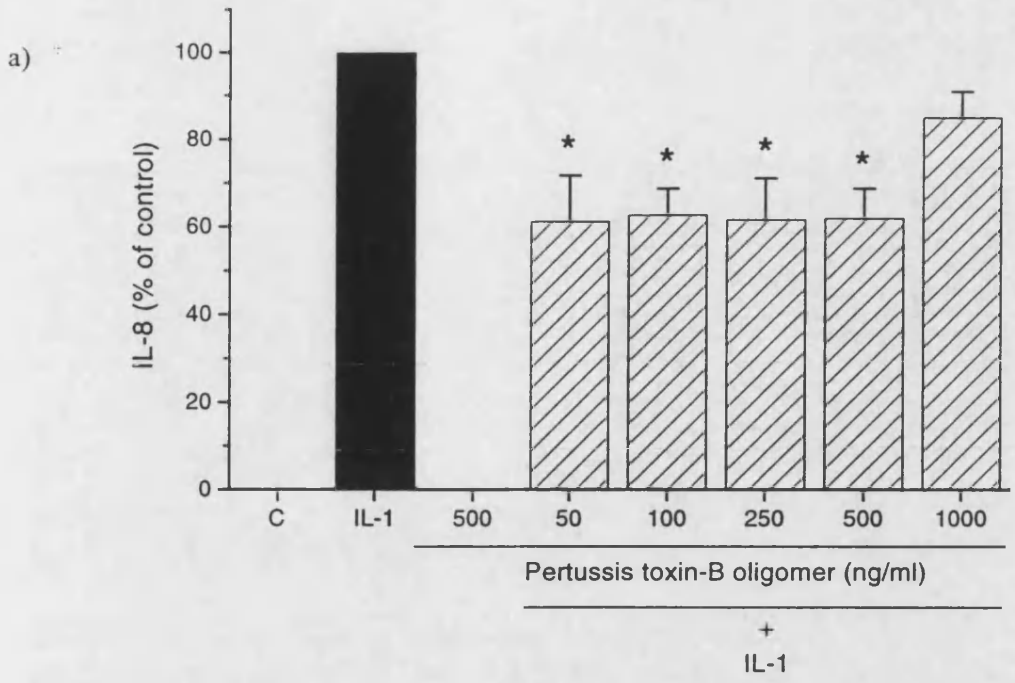


Figure 50. Pertussis toxin inhibits IL-1 induced IL-8 and IL-6 peptide production by MC. Cells cultured in 24 multi-well plates were pretreated with PT (50 - 1000 ng/ml) for 5 hours prior to the addition of a sub-maximal dose of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Control wells (C) were treated with media alone for 23 hours. Cell supernatants were quantitated for extracellular IL-8 (a) and IL-6 (b) peptide by ELISA. Results are expressed as a % of the IL-1α control, where cells were stimulated with IL-1α alone for 18 hours, and are the mean ± SEM of n=5.



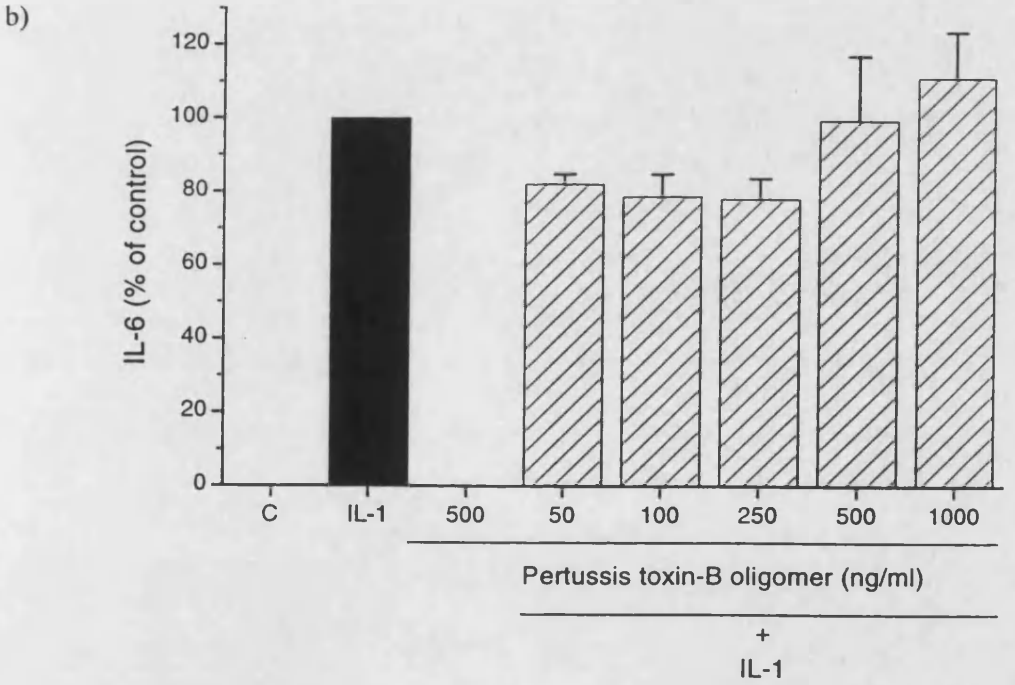


Figure 51. Effect of pertussis toxin B oligomer on IL-1 induced IL-8 and IL-6 peptide production. MC were pretreated with PT-B (50 - 1000 ng/ml) for 5 hours prior to the addition of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Cell supernatants were quantitated for extracellular IL-8 (a) and IL-6 (b) peptide by ELISA. Results are expressed as a % of the IL-1α control, where cells were stimulated with IL-1α alone for 18 hours, and are the mean ± SEM of n=5.

It was unexpected to find that such a marked biological effect for IL-8 was due to the binding subunit (PT-B), and was unconnected with the ADP-ribosyltransferase function of the toxin.

5.2.2. Ability of pertussis toxin and the B oligomer to ADP-ribosylate mesangial cell membrane proteins

ADP-ribosylation studies were therefore performed to confirm that PT was able to modify G protein substrates in MC membranes and to check that the preparation of PT-B used was infact devoid of ADP-ribosyltransferase activity. Membranes extracted from untreated MC, and treated with 10 μg/ml PT resulted in radiolabelled proteins of ~40 kDa (Fig. 52, lane 2). This correlates with the molecular weight of known isoforms of G_i (40 - 41 kDa) and G_0 (39 kDa) proteins (Milligan, 1988). The ~40 kDa band of radiolabelled proteins may consist of more than one type of PT-sensitive G protein which were not individually resolved using a 12.5% polyacrylamide gel. The ~26 kDa radiolabelled band (Fig. 52, lane 2) is non-specific, and due to auto-ribosylation of the S1 subunit (Kaslow & Burns, 1992). In comparison, incubation of MC membranes with 100 μg/ml PT-B instead of PT failed to generate radiolabelled bands (Fig. 52, lane 3), indicating that the PT-B preparation contained no detectable contamination with S1 subunit, and thus was devoid of ADP-ribosyltransferase activity.

To determine the time required for PT modification of the membrane pool of G protein substrates, cells were treated for 0, 1, 2 or 6 hours with PT (500 ng/ml). Membranes were then prepared and a second exposure of 15 minutes to PT in the presence of [^{32}P]-NAD was carried out. The intensity of the labelling by [^{32}P], visualised by autoradiography, would therefore be inversely related to the amount ADP-ribosylated during the initial incubation of the cells with the toxin. Figure 53 shows that after 1 hours incubation of cells with PT (lane 3), 39 ± 10% (mean ± SEM, n=3) of the membrane pool of PT-sensitive G proteins had been ADP-ribosylated, with 100% ribosylation observed at 6 hours (lane 5). It was therefore concluded that the conditions used to determine the effect of PT on IL-1α induced IL-8 and IL-6 production (5 hour pretreatment) would result in near total ribosylation, and inhibition of PT-sensitive G proteins.

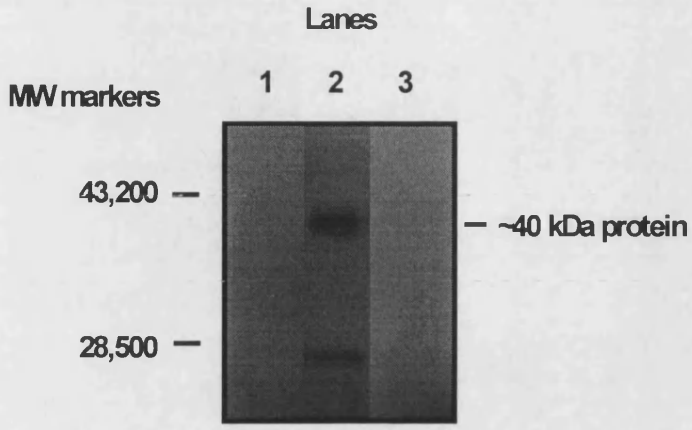


Figure 52. ADP-ribosylation of proteins in MC membranes by pertussis toxin or the B oligomer. Membranes were extracted from untreated MC cultured in 80 cm^2 flasks. 60 µg of membrane protein was exposed to PBS (lane 1), 10 µg/ml of pre-activated PT (lane 2) or 100 µg/ml pre-activated PT-B (lane 3) in the presence of [^{32}P]- NAD for 15 mins at 30°C. Membrane proteins were resolved by electrophoresis in a 12.5% SDS-polyacrylamide gel and autoradiographed. The relevant part of the autorad is shown, n=2.

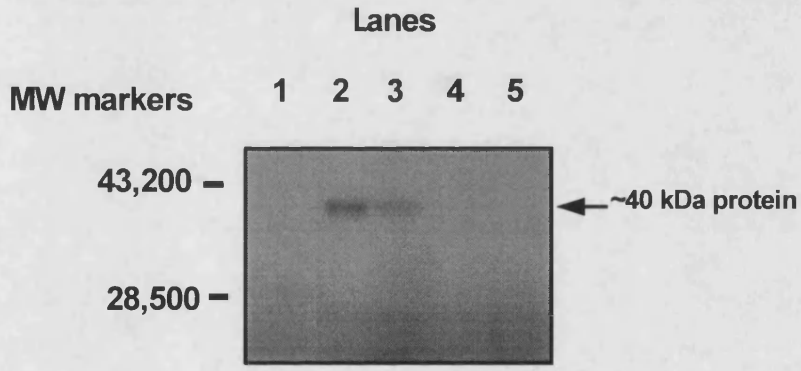


Figure 53. Time course of G protein modification by pertussis toxin in MC. MC cultured in 80 cm^2 flasks were treated with media alone (lanes 1 and 2) or 500 ng/ml PT for 1 hour (lane 3), 2 hours (lane 4) or 6 hours (lane 5). Membranes were then extracted and 60 µg of membrane protein re-exposed to 10 µg/ml of pre-activated PT (lanes 2-5) in the presence of [^{32}P] NAD for 15 mins at 30°C. Untreated membranes were also exposed to [^{32}P]-NAD in the absence of toxin (lane 1). Membrane proteins were resolved in a 12.5% SDS-polyacrylamide gel and autoradiographed. In lanes 3-5, the degree of G protein ribosylation that occurred on the initial exposure of intact cells to PT is inversely related to the amount of ribosylation observed on the autorad, which occurred during the second exposure of the cell membranes to PT in the presence of a [^{32}P]-NAD label. The relevant part of the autorad is shown and is representative of 2 other experiments.

5.2.3. Effect of pertussis toxin mutants on IL-1 induced IL-8 and IL-6 production

As both PT and PT-B were equally inhibitory for IL-1α induced IL-8 production, studies were carried out using mutants of PT to ascertain the relevant subunits of PT which were responsible for the inhibition. PT cross reactant mutants, PTR_9E and PTR_9ENK, were employed. PTR_9E contains point mutations in the S1 subunit and exhibits undetectable ADP-ribosylating activity, while PTR_9ENK contains point mutations in both the S1 and B oligomer subunits and has impaired ADP-ribosylating and binding activity (Lobet *et al.* 1993; Pizza *et al.* 1989; Loosemore *et al.* 1990).

Preliminary data indicated that pretreatment of MC with PTR_9E (50 to 1000 ng/ml) for 5 hours prior to the addition of IL-1α resulted in partial inhibition of IL-8 production, 31 ± 0.3% (mean ± SEM, n=1) inhibition was observed with 50 ng/ml PTR_9E (Fig. 54a). The inhibitory effect of PTR_9E on IL-1α induced IL-8 production was not dose-related between 50 and 500 ng/ml, the higher dose of 1000 ng/ml had no inhibitory effect. In contrast, PTR_9E (50 to 1000 ng/ml) had no clear effect on IL-1α induced IL-6 peptide production (Fig. 54b). These results imply that the ADP-ribosyltransferase activity of PT is not involved in the inhibitory effects of the toxin on IL-8 production.

Interestingly PTR_9ENK, which has both reduced ADP-ribosylating and binding activity resulted in inhibition of IL-1α induced IL-8 and IL-6 production at 50 to 500 ng/ml, and this was not dose related (Fig 55 a and b). 50 ng/ml of PTR_9ENK decreased IL-8 and IL-6 production by 32 ± 6% and 33 ± 1.4% (mean ± SEM, n=1) respectively, in response to IL-1α. No inhibition of IL-8 or IL-6 production was observed when a higher dose of PTR_9ENK was used.

Treatment of MC with PTR_9E or PTR_9ENK (500 ng/ml) for the duration of the experiment had no effect on basal IL-6 or IL-8 production (Fig. 54 and 55).

These latter results indicate that neither the binding of PT-B to specific cell surface receptors nor the ADP-ribosyltransferase activity are required for the pertussis toxin inhibition of IL-1α induced IL-8 or IL-6 production in MC.

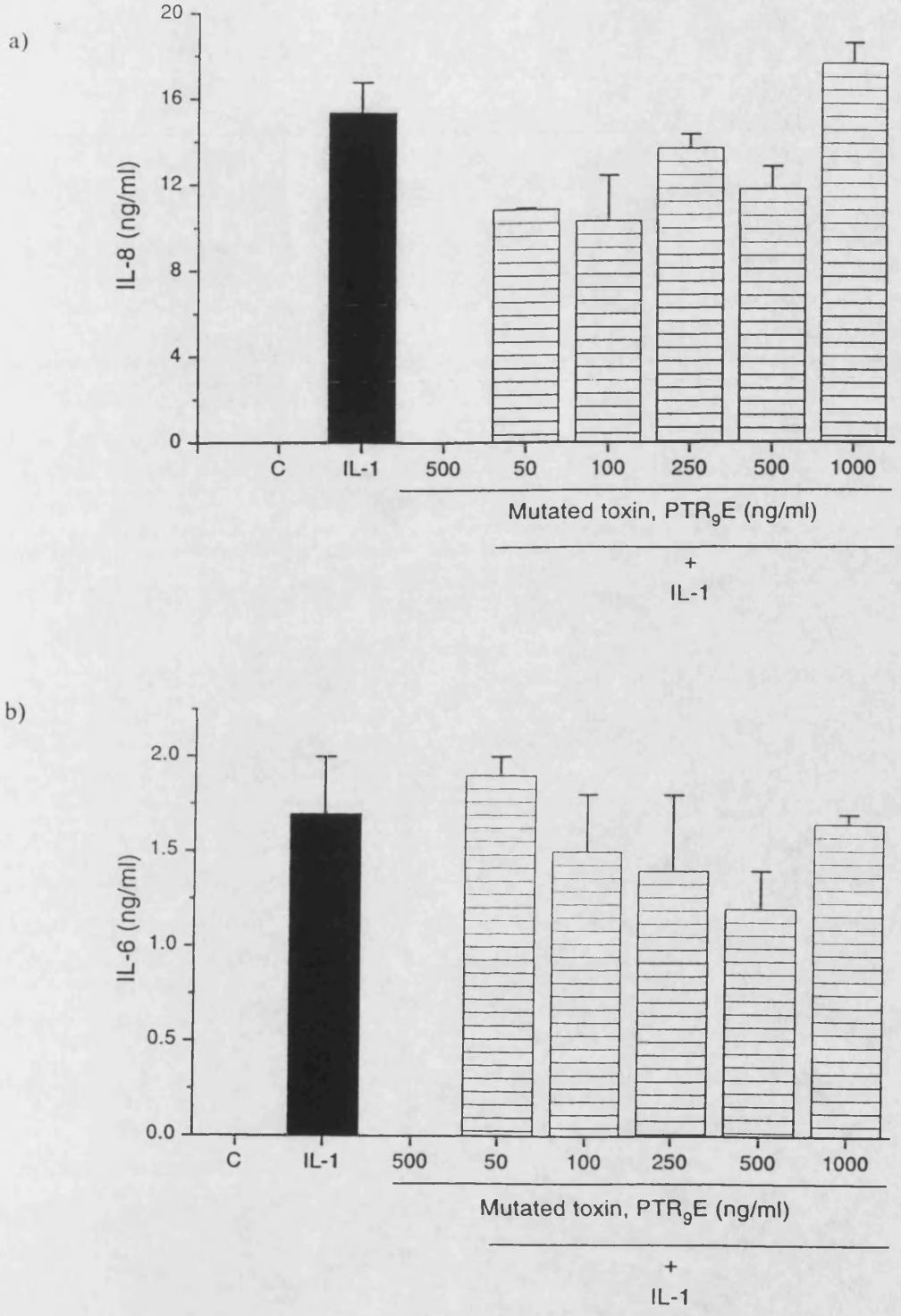


Figure 54. Effect of pertussis toxin mutant, PTR_9E (S1 subunit mutations) on IL-1 induced IL-8 and IL-6 peptide production. MC cultured in 24 multi-well plates were pretreated with PTR_9E (50 - 1000 ng/ml) for 5 hours prior to the addition of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Cell supernatants were quantitated for extracellular IL-8 (a) and IL-6 (b) peptide by ELISA. Results are the mean ± SEM of duplicate samples of n=1.

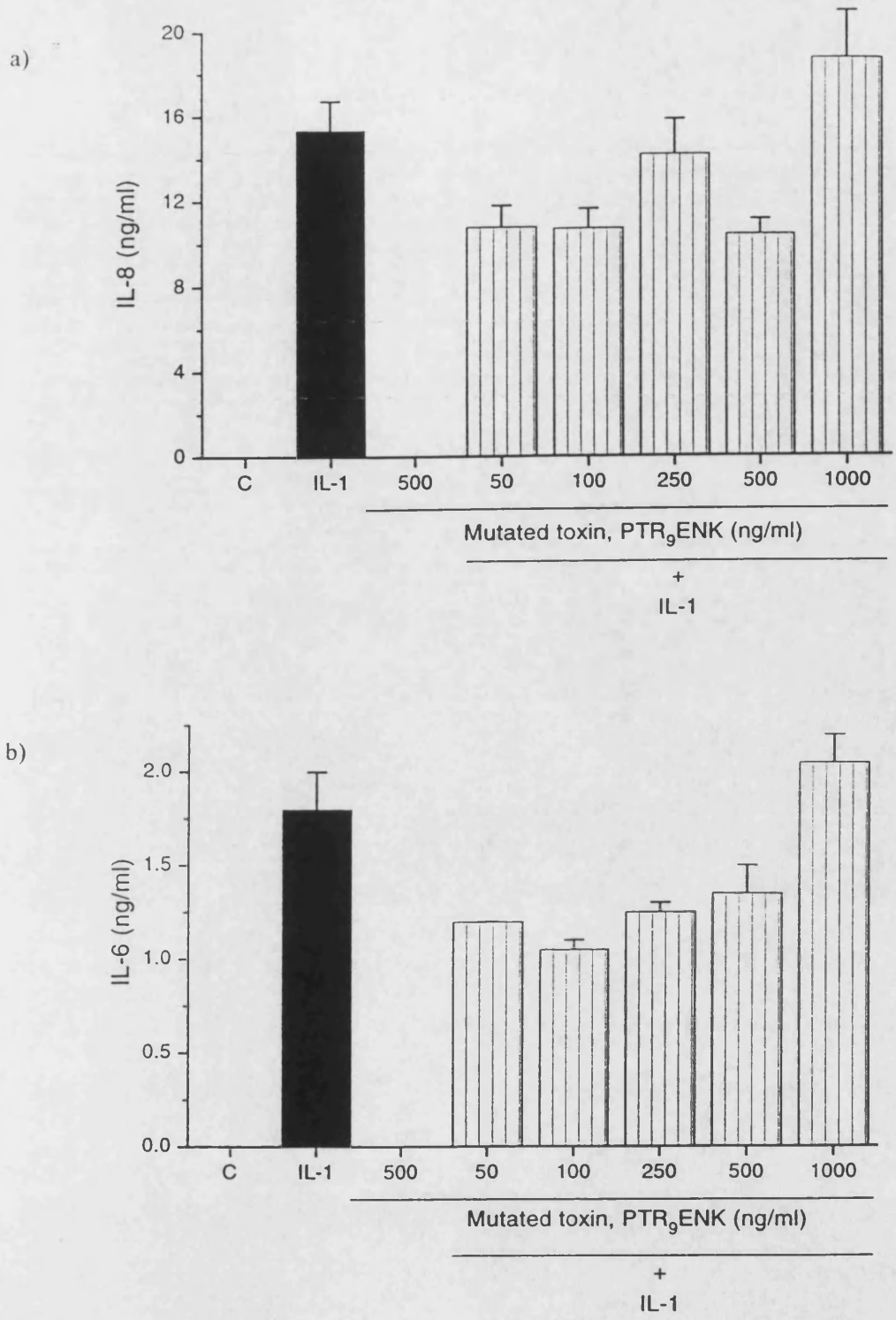


Figure 55. Effect of pertussis toxin mutant, PTR_9ENK (S1 and B oligomer mutations) on IL-1 induced IL-8 and IL-6 peptide production. MC cultured in 24 multi-well plates were pretreated with PTR_9ENK (50 - 1000 ng/ml) for 5 hours prior to the addition of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Cell supernatants were quantitated for extracellular IL-8 (a) and IL-6 (b) peptide by ELISA. Results are the mean ± SEM of duplicate samples from n=1.

5.2.4. Effect of recombinant pertussis toxin B oligomer on IL-1 induced IL-8 and IL-6 production

To further clarify the mechanism of PT and PT-B inhibition of IL-1α signalling for IL-8 and IL-6 production, a recombinant form of PT-B was employed. Recombinant PT-B is devoid of S1 subunit, and any other contaminating bacterial product derived from *Bordella pertussis*.

Pretreatment of MC with recombinant PT-B (50 to 1000 ng/ml) for 5 hours prior to the addition of IL-1α had no significant effect on IL-8 or IL-6 production (Fig. 56a and b). These findings confirm the B oligomer of PT is not responsible for the inhibitory effects of PT on IL-1α signalling events in MC.

Taken together, these results suggest that the inhibitory effects of non-recombinant PT-B, PTR_9E and PTR_9ENK may either be due to: (1) low level contamination of the preparations with active S1 subunit, which is not detected by *in vitro* ADP-ribosylation assays (see results section 4.2.3 and Pizza *et al.* (1989) and Loosemore *et al.* (1990)) or (2) the effects of a contaminating product of *Bordella pertussis*, which co-purifies with the toxin preparations.

5.2.5. Investigation of the ability of IL-1 or TNF to increase the cholera toxin ribosylation of a pertussis toxin-sensitive G protein

As the mechanism of pertussis toxin inhibition of IL-1α induced IL-8 and IL-6 production is unclear, and may be independent of G protein modification by ADP-ribosylation, a different method was employed to probe MC for a direct IL-1α or TNFα receptor-$G_{i/o}$ type protein interaction.

In the absence of guanine nucleotides in the ADP-ribosylation reaction mixture, CT additionally labels a ~40 kDa protein in mesangial cell membranes, which normally only serves as a substrate for PT (Fig. 57). An ability of agonists to cause a specific increase in the CT labelling of the ~40 kDa protein has been shown to indicate a direct coupling between the agonist receptors and $G_{i/o}$-type proteins (Milligan *et al.* 1991; Iiri

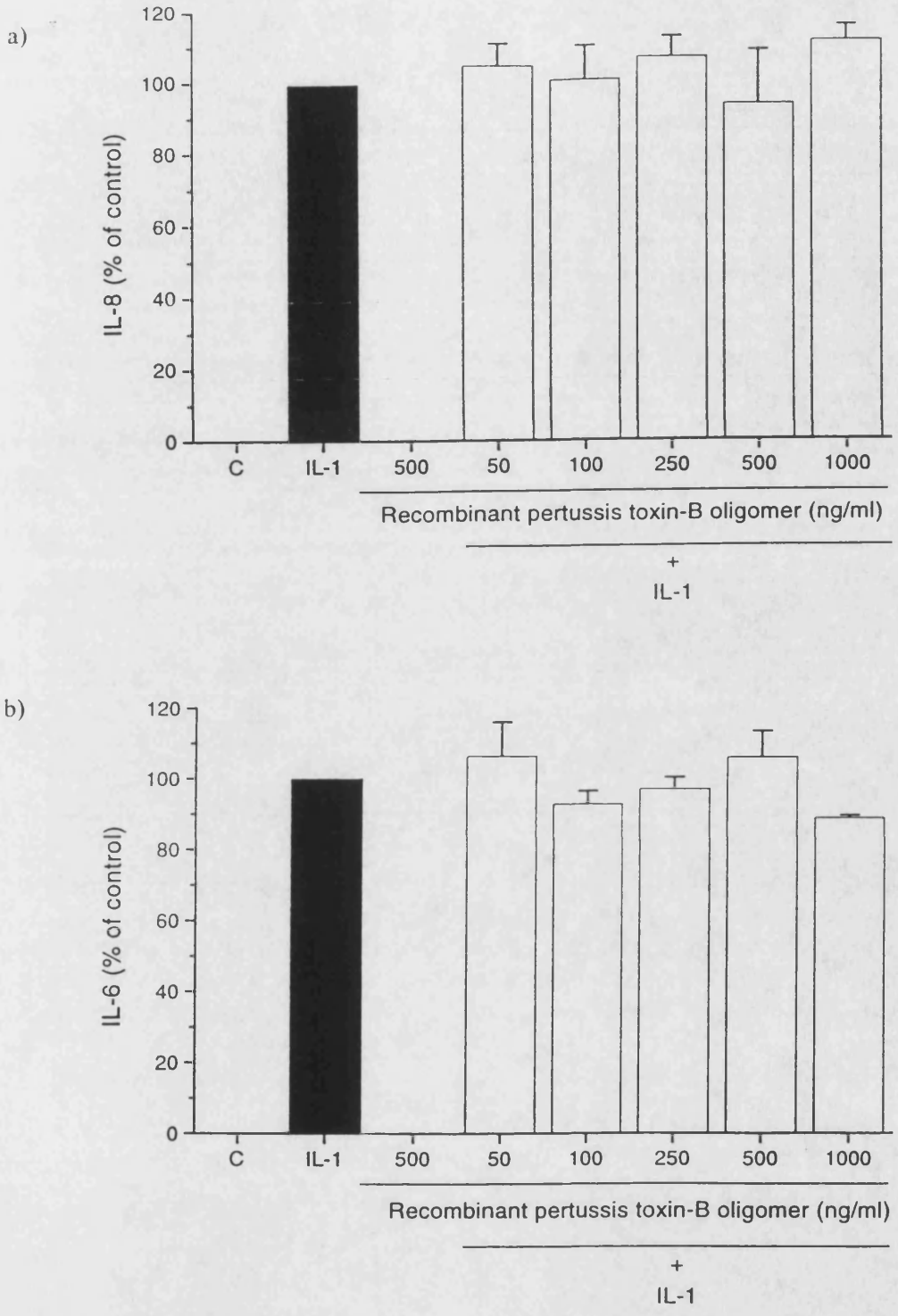


Figure 56. Inability of recombinant pertussis toxin B oligomer to modify IL-1 induced IL-8 and IL-6 peptide production. MC were pretreated with recombinant PT-B (50 - 1000 ng/ml) for 5 hours prior to the addition of IL-1α (3 ng/ml) to the drug containing media for 18 hours. Cell supernatants were quantitated for extracellular IL-8 (a) and IL-6 (b) peptide by ELISA. Results are expressed as a % of the IL-1α control, where cells were stimulated with IL-1α alone for 18 hours, and are the mean ± SEM of n=3.

et al. 1989). The mechanism thought to account for this phenomena is that GDP must be released from the guanine nucleotide pocket of the $G_{i/o}$-type protein by the activated agonist/receptor complex to provide a form of the protein which is susceptible to CT ribosylation. Figure 58 shows 1 or 3 μg/ml IL-1α (lanes 2 and 3), and 1 or 10 μg/ml TNFα (lanes 4 and 5) failed to cause any observable increase in the labelling of the ~40 kDa band. Endothelin-1 (3 μM), included as a positive control was also without effect (Fig. 58, lane 6), which indicates the direct coupling of endothelin receptors to PT-sensitive G proteins in MC (Thomas *et al.* 1991) could not be detected under the experimental conditions employed. The inclusion of 2.5 mM Mg^{2+} or 17 mM EDTA in the ADP-ribosylation assay had no effect on labelling of the ~40 kDa band in this study (data not shown). Both these conditions were tried due to contrasting reports in the literature on the requirement for Mg^{2+} during the ADP-ribosylation assay. One study reported Mg^{2+} was necessary for GDP release from the G protein (Iiri *et al.* 1989), while a separate study found Mg^{2+} only served to increase background (agonist independent) labelling of the ~40 kDa substrate (Milligan *et al.* 1991). Radiolabelled bands at ~60, ~70 (Fig. 58) and ~21 kDa (not shown) are thought due to non-specific labelling. The ~70 kDa band corresponds to the MW of BSA present in the IL-1α vehicle, the intensity of labelling increased when higher doses of IL-1α were used. Labelling of BSA present in the TNFα vehicle was not evident due to higher stock concentrations, which therefore required greater dilution for use. The ~21 kDa band corresponds to autoribosylation of the S1 subunit of CT (Mekalanos, 1988).

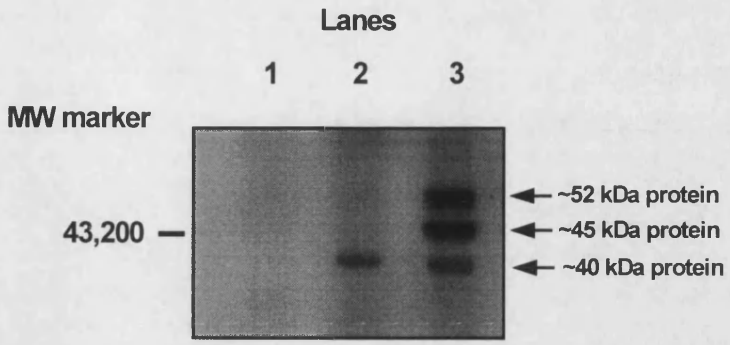


Figure 57. Ribosylation of a ~40 kDa pertussis toxin-sensitive G protein in MC membranes following incubation with cholera toxin in the absence of guanine nucleotides. Membranes were extracted from untreated MC cultured in 80 cm^2 flasks. 60 µg of membrane protein was exposed to PBS (lane 1), 10 µg/ml pre-activated PT (lane 2) or 50 µg/ml pre-activated CT (lane 3) in the presence of [^{32}P]-NAD for 15 mins at 30°C. Guanine nucleotides were omitted from the reaction mixture throughout. Membrane proteins were resolved on a 12.5% SDS-PAGE gel and autoradiographed. The relevant part of the autorad is shown, n=1.

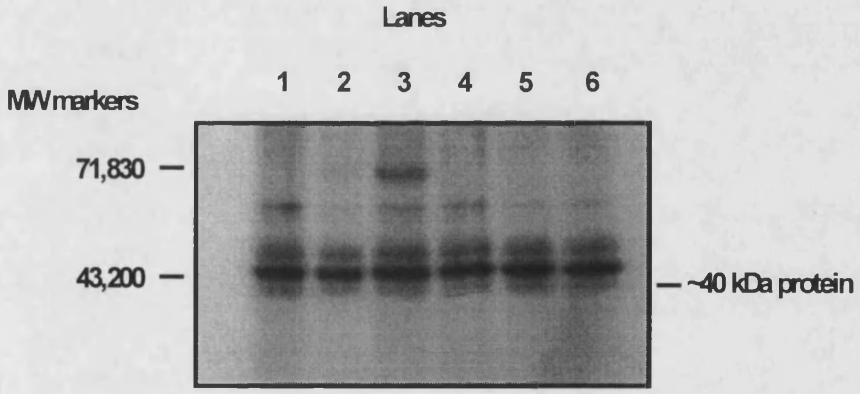


Figure 58. Inability of IL-1, TNF or endothelin-1 to increase the cholera toxin ribosylation of a ~40kDa G protein. Membranes were extracted from untreated MC cultured in 80 cm^2 flasks. 60 µg of membrane protein was exposed to 50 µg/ml pre-activated CT (lane 1-6) in the presence of media (lane 1), 1 or 3 µg/ml IL-1α (lanes 2 and 3 respectively), 1 or 10 µg/ml TNFα (lanes 4 and 5 respectively) or 3 µM endothelin-1 (lane 6) for 1 hour at 30°C. Guanine nucleotides were omitted from the reaction mixture throughout. Membrane proteins were resolved on a 12.5% SDS-PAGE gel and autoradiographed. The relevant part of the autorad is shown, which is representative of 2 other experiments.

5.3. DISCUSSION

Pertussis toxin has become an important experimental tool for investigating G protein involvement in signal transduction mechanisms. However, its biological effects may not be solely due to its ability to modify G proteins.

The present study investigated the inhibitory effect of pertussis toxin on IL-1 induced IL-8 and IL-6 production in human MC. Treatment with the toxin resulted in partial inhibition of IL-1 induced IL-8 (40%) and IL-6 (27%) generation. No further inhibition of the IL-1 response was observed following treatment with higher doses of the toxin, which may reflect saturation with PT by 50 ng/ml. Furthermore, ADP-ribosylation studies demonstrate that 2 hours contact of MC with toxin resulted in almost complete ribosylation of the susceptible G protein pool, with no further ribosylation observed after 6 hours contact. It was therefore concluded that the conditions in which the toxin partially inhibited IL-8 and IL-6 production (5 hours) would have resulted in near total ADP-ribosylation and inactivation of $G_{i/o}$ type proteins. This latter finding is consistent with previous studies showing PT modified the susceptible pool of G proteins in cultured cells within a 4 hour incubation (Katada *et al.* 1983). An inhibitory effect of PT on IL-1 induced IL-8 generation has been previously reported in the human monocytic cell line, U937 (Pleass & Westwick, 1991), pretreatment with 100 ng/ml PT resulted in 80% inhibition of IL-8 generation. In contrast, PT was found to have no inhibitory effect on IL-1 induced IL-6 production in human gingival fibroblasts (O'Neill *et al.* 1992). The inhibition of IL-6 production by PT observed in the present study was not as marked as the effects on IL-8 generation.

The characterized action of PT, the inactivation of $G_{i/o}$ type proteins through ADP-ribosylation appeared not to be responsible for the observed inhibitory effect of PT on IL-1 induced IL-8 or IL-6 production in MC, as the purified B oligomer of PT (PT-B), which was devoid of ADP-ribosylating activity was found to be equally inhibitory. The partial inhibition of IL-1 induced IL-6 production by PT-B was significant ($P<0.01$) at the level of ANOVA, but did not reach statistical significance when further analysed using the Dunnetts t test. The relatively small level of inhibition of IL-1 induced IL-6 production by both PT and PT-B treatment may account for these statistical findings.

Possible contamination of the PT-B preparation with S1 subunit is unlikely to account for its' inhibitory effects on IL-8 and IL-6 production, as PT-B contained undetectable ADP-ribosyltransferase activity when incubated with MC membranes at 10 times the effective concentration of PT. These findings support the manufacturers claim that the PT-B preparation contained less than 0.02% contamination with holotoxin. The flat dose response curve for IL-8 and IL-6 inhibition by PT-B may reflect saturation by 50 ng/ml. The similarity between the dose-response curves for holotoxin and B oligomer further suggest that contamination of the PT-B preparation with S1 subunit was not responsible for its' inhibitory activity, as PT-B was as effective as the holotoxin across the dose range employed. Unfortunately, the range of doses employed did not extend low enough to identify the minimum amount of PT or PT-B required for an inhibitory effect.

Of relevance to the present findings is a recent study by O'Neill *et al* (1992), which investigated the PT inhibition of IL-1 induced IL-2 secretion in EL4 cells and PGE_2 secretion in human gingival fibroblasts. In both these cells the purified B oligomer was as inhibitory as the holotoxin. Furthermore, the inhibition by PT and PT-B was found to be specific for the IL-1 pathway, as PT-B did not impair the ability of PMA to induce IL-2 or PGE_2 generation in the respective cell types. PT-B appeared to be inhibiting at a later point in the IL-1 pathway, as the affinity or number of IL-1 receptors was not altered and PT-B did not inhibit an early response of EL4 cells to IL-1, induction of the transcription factor NFκB. These findings support the observations in MC that inhibition of certain IL-1 activities by PT is attributable to unknown actions of the toxin which appear independent of G protein modification by ADP-ribosylation. In direct contrast to the report by O'Neill *et al* is a study performed in a different subclone of EL4 cells which found the inhibition of IL-1 induced IL-2 secretion by PT was not mimicked by the purified B oligomer (Rollins *et al.* 1991). The reason for the discrepancy between these two studies is at present unclear. Unfortunately other studies demonstrating an effect of PT on IL-1 signalling events, including PGE_2 production in human synovial cells, kIg light chain expression by 70Z/3 cells, IL-2R expression by YT cells and DAG production in EL4 cells, did not investigate the effects of purified PT-B (Chedid *et al.* 1989; Dobson *et al.* 1989).

The study by O'Neill *et al* (1992) concluded that the ability of PT to inhibit certain IL-1 activities was attributable to unknown actions of the B oligomer. Signalling properties of purified PT-B have been previously described. PT-B stimulated mitosis in murine splenocytes and human T lymphocytes, and enhance glucose oxidation in rat adipocytes (Tamura *et al.* 1983; Strnad & Carchman, 1993). The mechanism of PT-B action appeared to involve a rapid and sustained elevation of cytosolic Ca^{2+} predominantly from extracellular stores (Strnad & Carchman, 1993). The lectin concanavalin A (con A) exhibited similar effects to PT-B in these studies. The activities of con A are thought due to its' multipoint attachment to cell surface glycoproteins resulting in the aggregation of receptor proteins and subsequent changes in cytosolic Ca^{2+}. Aggregation of receptor molecules by PT-B may therefore be important in mediating its effects, in an analogous manner to con A (Strnad & Carchman, 1993; Tamura *et al.* 1983). The structural properties of PT-B are consistent with such a model, consisting of the two dimers, S2-S4 (dimer 1) and S3-S4 (dimer 2), connected by S5. Interaction of each dimer with an independent recognition site would result in a bivalent binding phenomenon. The effects of PT or PT-B on lymphocyte proliferation, glucose oxidation and intracellular Ca^{2+} levels were only observed when high doses of the toxin were employed (≥0.5 μg/ml). The marked difference in the dose-response of PT-B inhibition of IL-1 induced IL-8 and IL-6 production in human MC, and IL-2 production in EL4 cells (where 100 ng/ml PT-B was used) (O'Neill *et al.* 1992), indicates the mechanism of regulation may differ, and therefore elevation of intracellular Ca^{2+} by PT-B may not be involved. Indeed, the results of the presents study obtained using a PT mutant, PTR_9ENK and a recombinant preparation of PT-B indicate that the properties of the B oligomer may not be important in mediating the inhibitory effects of the toxin on IL-1 activity.

The PT mutant, PTR_9ENK contained points mutations in the S1, S2 and S3 subunits. The mutations in the S1 subunit consisted of substitution of arginine 13 and glutamine 129, with leucine and glycine respectively. These substitutions in the S1 subunit have been shown to result in undetectable levels of ribosylation of the G protein, transducin by pertussis toxin in ADP-ribosylation studies and to cause complete attenuation of the *in vivo* activities of the toxin in mice, which are known to be mediated by its ADP-

ribosylating activity (Pizza *et al.* 1989; Loosemore *et al.* 1990). The mutations in the B oligomer were located in S2 and S3, as these subunits are thought to contain the receptor recognition sites, although the identity of the cellular receptors for PT have yet to be identified (Schmidt & Schmidt, 1989; Schmidt *et al.* 1991; Kaslow & Burns, 1992). Deletion of the asparagine residue 105 of S2 and the lysine residue 105 of S3 resulted in complete inhibition of the ability of PT to induce agglutination of red blood cells, cytotoxicity in Chinese hamster ovary (CHO) cells and mitogenic effects in T lymphocytes (Lobet *et al.* 1993), activities of PT that are known to be mediated by the B oligomer. These reports demonstrate the mutations in PTR_9ENK effectively inhibit activities of PT which are mediated by its' ADP-ribosylating activity or by properties of the B oligomer, however my preliminary results indicate PTR_9ENK was as effective as the holotoxin in inhibiting IL-1 induced IL-8 and IL-6 production in MC. This suggests neither the ADP-ribosyltransferase activity of the toxin nor the properties of the B oligomer are involved in the inhibition of IL-1 induced IL-6 and IL-8 production. The similarity between the dose response curves for holotoxin and PTR_9ENK inhibition of IL-8 and IL-6 production, with both toxins being inhibitory between 50 and 500 ng/ml, while a 1000 ng/ml dose was ineffective, argues against the possibility that residual S1 activity is responsible for the effects of PTR_9ENK. In addition, the inability of a recombinant preparation of PT-B to modify IL-8 or IL-6 production confirms the inhibitory effects of PT are not mediated by the B oligomer.

Taken together the findings of this study suggest the inhibitory activity of PT, non-recombinant PT-B and PTR_9ENK on IL-1 induced IL-8 and IL-6 production in MC may be due to contamination with a distinct product from *B. Pertussis* which co-purifies with the toxin, since only the recombinant form of PT-B, produced in a host other than *B. Pertussis,* showed no inhibitory activity. The mechanism of inhibition of IL-1 induced IL-8 and IL-6 production by pertussis toxin is therefore unknown, but appears independent of ADP-ribosylation and thus does not indicate a role for $G_{i/o}$ type proteins in IL-1 signalling for IL-8 and IL-6 in MC. While the effects of pertussis toxin preparations on certain IL-1 signalling events may prove to be interesting, until the mechanism of action is understood, the involvement of any specific signalling component cannot be inferred.

In the current literature, evidence for a G protein involvement in IL-1 and TNF signal transduction has also arisen from studies involving GTPγS binding experiments and GTPase assays (Chedid *et al.* 1989; O'Neill *et al.* 1990a; Imamura *et al.* 1988). These methods detect a direct functional interaction between the agonist receptor and heterotrimeric G proteins in cell membrane preparations. The ability of PT to inhibit the IL-1 and TNF stimulated increases in GTP hydrolysis suggests, in this system, that PT-sensitive G proteins such as G_i or G_o isoforms may be involved (Chedid *et al.* 1989; O'Neill *et al.* 1990a; Imamura *et al.* 1988).

A different experimental approach was therefore employed to probe mesangial cells for a direct IL-1 or TNF receptor-PT-sensitive G protein coupling. The novel technique used was based on the ability of an agonist to specifically increase the CT induced ADP-ribosylation of a ~40 kDa PT-sensitive G protein, if the agonist-receptor complex functionally coupled to that G protein (Milligan *et al.* 1991; Gierschik & Jakobs, 1987; Iiri *et al.* 1989). However, IL-1, TNF and endothelin-1 were found to have no observable effects in this system. Endothelin-1 was included as a positive control as its' receptor is a member of the family of G protein-coupled receptors which possess 7 membrane spanning domains, and endothelin is known to signal via a PT-sensitive G protein in MC (Thomas *et al.* 1991). A number of reasons may account for the inability of IL-1, TNF or endothelin-1 to induce an observable effect in this system. Firstly, this technique may be unsuitable for detecting all types of receptor-PT-sensitive G protein interactions. To date, this novel method has been used to demonstrate 2 types of receptor G protein interactions: coupling of the human platelet α_2-C10 adrenergic receptor to G_{i2} and G_{i3} proteins in rat-1 fibroblasts transfected with this receptor (Milligan *et al.* 1991), and coupling of the formyl-methionyl-leucyl-phenylalanine (FMLP) receptor to G_{i2} proteins in the neutrophil-like HL-60 cell line (Iiri *et al.* 1989; Gierschik & Jakobs, 1987). A more recent study in HL-60 cells investigated the ability of both FMLP and LTB_4 to specifically enhance the CT ribosylation of a PT-sensitive G protein (Schepers & McLeish, 1993). Both these agonists induce disparate second messengers and functional responses in HL-60 cells, but are thought to couple to a common pool of PT-sensitive G proteins, since both agonists increase GTP hydrolysis and GTPγS binding in cell membrane preparations, which was inhibitable by PT

treatment (McLeish *et al.* 1989). However only FMLP enhanced the CT ribosylation of a ~40 kDa substrate, while LTB_4 had no effect. It was therefore suggested that the PT-sensitive G proteins that couple to the FMLP and LTB_4 receptors exist in different activated conformations which is determined by the receptor with which they interact, and not all activated conformations may be susceptible to CT ribosylation. Additional doubts on the ability of an IL-1 or TNF receptor-G protein interaction to be detected by this method rests on the fact that like endothelin, both the FMLP and LTB_4 receptors belong to the family of classical G protein coupled receptors which possess the common structural feature of 7 membrane spanning domains. The nature of the G protein coupling occurring between receptors that do not belong to this family has yet to be established and may prove to be quite different (Ray *et al.* 1992).

A further consideration is the relatively low number of IL-1 and TNF receptor expressed on cells (200 - 4000 IL-1 receptors (Saklatvala & Guesdon, 1992) and 100 - 10,000 TNF receptors (Beyaert & Fiers, 1994)) compared to the number of high affinity FMLP receptors on HL-60 cells (>46,000) (Weisbart *et al.* 1986), the model cell system for demonstrating the use of this method in showing a receptor-G protein interaction. It is therefore possible that the number of IL-1 and TNF receptors present on MC is insufficient, even if the majority are activated, to cause an observable increase in ribosylation over the relatively high basal labelling that occurs. The other cell system in which this method has been successfully employed is rat-1-fibroblasts transfected with the α_2-C10 adrenergic receptor, and in this study, only clones showing high receptor expression were used (Milligan *et al.* 1991). Furthermore, in the previous studies (Iiri *et al.* 1989; Gierschik & Jakobs, 1987) high concentrations of FMLP (≥100 nM), which far exceed the concentrations necessary to elicit a biological response (1 nM) (Cassatella *et al.* 1992) were required to induce an observable effect, indicating that a large number of receptors need to be activated. The lack of effect in the MC system should not simply be a question of insufficient receptor occupancy with ligand, since high concentrations of all 3 agonists were tried (upto 3 μg/ml IL-1α (180 nM), upto 10 μg/ml TNFα (590 nM) and 3 μM endothelin-1).

Finally, the possibility that the experimental conditions employed in this study were not correct for supporting an agonist dependent CT ribosylation of a PT-sensitive G protein cannot be ruled out, due to the absence of an appropriate positive control. Based on the points discussed above, the most appropriate positive control to use would be the HL-60/FMLP system, since there are no doubts concerning the ability of FMLP to induce an observable effect using this technique. Such a control would have confirmed viable membrane preparations with intact receptors were obtained after the extraction procedure, and that the conditions for ADP-ribosylation were correct.

The techniques employed in the present study proved to be unsuitable for determining whether IL-1 or TNF signal via PT-sensitive G proteins in human MC. The low number of IL-1 receptors present on cells is likely to pose a problem in many of the current techniques available for identifying a receptor-G protein interaction. GTPase assays and GTPγS binding studies are the most widely employed techniques for identifying G protein coupled receptors, and both methods were used to demonstrate an IL-1 receptor-G protein interaction in 7OZ/3 cells (Chedid *et al.* 1989) and an EL4 cell line (O'Neill *et al.* 1990a). In contrast, a subsequent study using both the 70Z/3 and EL4 cell lines failed to demonstrate any such effects of IL-1 using these techniques (Ray *et al.* 1992). Such discrepancy may be due to the fact a specific IL-1 receptor rich strain of EL4 cells (NOB-1) was used in the initial study (O'Neill *et al.* 1990a), while the latter investigation employed a different strain of EL4 cells (EL4.6.1.d10) (Ray *et al.* 1992). Furthermore, problems in reproducing these findings may be due to the fact the number of IL-1 receptors present on cells is greatly exceeded by the number of GTP binding sites, and therefore unless agonist-bound IL-1 receptors can couple G proteins with an exceptionally high frequency, significant IL-1 induced changes in the rate of GTP binding or hydrolysis in membrane preparations will be difficult to measure above the high backgrounds observed when using these techniques (Ray *et al.* 1992). Similar considerations concerning receptor number may apply to investigations into a TNF receptor-G protein coupling.

6. DETERMINATION OF THE POTENTIAL OF HUMAN MESANGIAL CELLS TO GENERATE NITRIC OXIDE

6.1. RATIONALE FOR STUDY

At the time of starting this area of work, reports describing production of nitric oxide (NO) by any human cell type were very limited. In contrast, a number of studies had demonstrated activation of the NO pathway in rodent mesangial cells by proinflammatory stimuli such as IL-1β, TNFα or LPS (Pfeilschifter & Schwarzenbach, 1990; Shultz *et al.* 1991). Previous studies in our laboratory had indicated the NO/cGMP pathway may play a role in the IL-1 regulation of IL-8 production in human MC (Brown *et al.* 1993b). These observations suggest that iNOS may be induced in cytokine activated human MC and may regulate chemokine expression via NO induced elevation of intracellular cGMP. Inorder to explore further the potential role of the NO pathway as a novel signal transduction mechanism for chemokine regulation, it was important to demonstrate NO production by cytokine activated human MC *in vitro* in the first instance.

6.2. RESULTS

6.2.1. Ability of cytokine activated human mesangial cells to generate nitrite

NO production by human mesangial cells was determined by measuring in cell supernatants the stable end-product nitrite using the Griess reaction. Incubation of growth-arrested MC in 24 multi-well tissue culture plates (~$5x10^4$ cells/ml/well) with Waymouths medium alone for 72 hours resulted in undetectable nitrite levels (<0.8 μM). The proinflammatory cytokines IL-1α (10 ng/ml) or IFN-γ (50 to 500 U/ml) added alone or in combination failed to induce detectable nitrite levels after 24, 48 or 72 hours (n=2, data not shown).

A number of modifications were therefore introduced to improve the system for detecting nitrite levels in MC culture supernatants;

(1) Nitrite levels were quantitated using a fluorimetric nitrite assay, which has a lower detection limit of ~100 nM. This compares to 0.8 μM, the detection limit of the Griess reaction.

(2) MC were grown in 25 cm^2 tissue culture flasks (~$8x10^5$ cells/flask), and stimulated with cytokines in a final volume of 3 ml. This enhanced the sensitivity of the system by increasing the cell:volume ratio. Flasks were also employed as a larger volume of supernatant (2 ml) was required for the measurement of undiluted sample using the fluorimetric assay.

(3) MC were not growth-arrested prior to the start of the experiments, in case this reduced the magnitude of the cytokine induced NO response, as was previously observed for TNFα induced IL-6 production in human MC (see chapter 1).

(4) IL-1β, as opposed to IL-1α was employed, as the majority of published reports demonstrating nitrite generation by either animal or human cells in culture used this isoform of IL-1 (Nussler *et al.* 1992; Junquero *et al.* 1992; Pfeilschifter *et al.* 1992).

The results obtained after the introduction of these modifications were as follows:- supernatants from mesangial cells incubated with media alone for 48 hours contained nitrite levels of 90 ± 22 nM (mean ± SEM, n=8) (data not shown). Stimulation of MC with IL-1β (0.3 to 10 ng/ml), IFN-γ (100 U/ml) or TNFα (10 to 300 ng/ml) alone failed to increase nitrite generation above media control levels after 24 or 48 hours (n≥3). Similarly, MC stimulated with a combination of IL-1β (0.3 to 10 ng/ml) and IFN-γ (10 to 500 U/ml), or IL-1β (0.3 to 10 ng/ml), IFN-γ (10 to 500 U/ml) and TNFα (10, 30 ng/ml) for 24 or 48 hours showed no generation of nitrite above basal levels (n≥3). The addition of 10 μg/ml LPS or 100 μM Db-cAMP to a cytokine cocktail of IL-1β (1 ng/ml), IFN-γ (100 U/ml) and TNFα (10 ng/ml), or to IL-1β (1 ng/ml) alone had no effect on nitrite levels after 48 hour (n=2).

Further changes to the methodology were tried;

(1) MC were stimulated with cytokines in the presence of 10% FCS to determine whether serum factors were required for the induction of iNOS activity.

(2) Experiments were performed on freshly isolated MC cultures at passage 2 and above, to assess whether long term culturing of MC or the storage of cultures in liquid nitrogen was leading to phenotypic changes which resulted in loss of the ability of MC to produce NO.

(3) Culture supernatants were assayed immediately after the experiment as opposed to storing at -20°C, to ensure that any nitrite present was not being broken down during storage.

(4) Experiments were performed in a different medium, Dulbecco's Modified Eagles Medium (DMEM) without phenol red, as previous studies reported that phenol red interfered with both the Griess reaction and fluorimetric assay (Green *et al.* 1982; Misko *et al.* 1993).

(5) MC were grown in 80 cm^2 tissue culture flasks (~$2.5x10^6$ cells/flask) and stimulated with cytokines in a final volume of 6 ml for 72 hours, to further enhance sensitivity by increasing the cell:volume ratio.

(6) The arginine content of Waymouths medium was increased from 360 μM to 2 mM during the experiment, incase substrate levels were limiting.

The above changes to the system had no effect on the ability of the cytokines IL-1β, TNFα or IFN-γ, alone or in combination, to increased nitrite levels in MC culture supernatants above basal levels after 24, 48 or 72 hours stimulation ($n \geq 2$, data not shown).

As a positive control, rat kidneys were obtained from untreated male Sprague Dawley rats and mesangial cells were isolated and cultured in a similar manner to human mesangial cells. Rat MC grown in 25 cm^2 tissue culture flasks were used at passage 2 and 3, and the experiment was performed in serum-free Waymouths medium on cultures which were not growth-arrested prior to use. Supernatant from rat MC incubated with medium alone for 24 or 48 hours contained undetectable nitrite levels (<0.8 μM) using the Griess reaction. In comparison, stimulation of rat MC with IL-1β alone (0.3 to 10

ng/ml) for 24 or 48 hours resulted in dose-dependent nitrite generation, 22 μM and 33 μM nitrite was detected after 24 and 48 hours stimulation with 3 ng/ml IL-1β respectively (Fig. 59). The results indicate that the stock of IL-1β and the Griess assay were functional, and performing the experiments in Waymouths medium in the absence of FCS supported nitrite generation by cultured cells. The successful detection of increased nitrite levels in culture supernatants from the human colonic epithelial cell line HT-29 in our laboratory, following stimulation with a combination of IL-1α, IFN-γ and TNFα using the fluorimetric nitrite assay confirmed this latter assay and stock cytokines were also functional (Kolios *et al.* 1995).

Together, these findings indicate that cytokine stimulation of human MC resulted in undetectable increases in nitrite levels of culture supernatants, using both the Griess (<0.8 μM) and fluorimetric (<100 nM) nitrite assays.

6.2.2. Ability of cytokine activated human mesangial cells to produce nitrate

Contrasting reports on the fate of NO in aqueous solution describe either oxidation of NO to nitrite and nitrate in a 2:1 ratio (Marletta *et al.* 1988), or complete oxidation of NO to nitrite, with little or no nitrate formed (Ignarro *et al.* 1993). Nitrate, however may be formed if FCS is present in the culture medium during the experiment, as small amounts of haemoglobin present in FCS will result in the conversion of NO to nitrate (Misko *et al.* 1993). The ability of human MC to produce superoxide (O_2^-) following cytokine stimulation (Radeke *et al.* 1990) may also lead to nitrate rather than nitrite production, since NO and O_2^- react with high affinity to form the peroxynitrite anion, which degrades to nitrate (Mulligan *et al.* 1991). To determine whether the inability to detect increased nitrite levels in culture supernatants from cytokine stimulated MC was due to the oxidation of NO to nitrate as a result of superoxide generation or the presence of FCS in the system, nitrate levels in culture supernatants from the previous experiments (described in 6.2.1) were quantitated by incubating the sample with nitrate reductase, an enzyme which converts nitrate to nitrite for measurement by the Griess or fluorimetric nitrite assays.

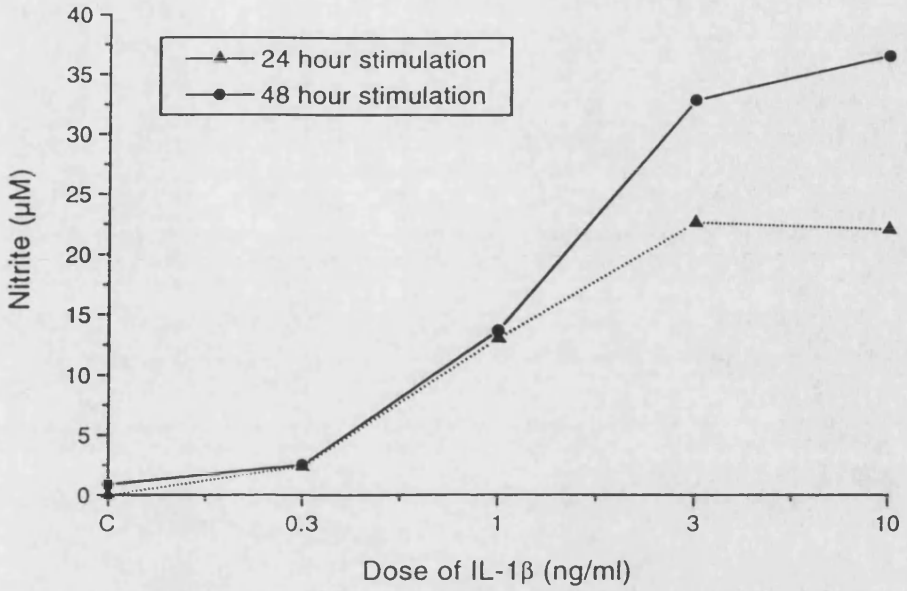


Figure 59. Dose-dependent production of nitrite by IL-1 activated rat MC. Sprague Dawley rat MC cultured in 25 cm^2 flasks were stimulated with increasing doses of IL-1β for 24 or 48 hours. Nitrite levels in cell supernatants were quantitated using the Griess reaction. A representative experiment is shown, similar nitrite production was observed in one other study.

Results obtained using the Griess reaction showed supernatants from MC incubated with media alone for 48 hour contained undetectable nitrite/nitrate levels. MC stimulated with IL-1β (0.3 to 3 ng/ml) or TNFα (300 ng/ml) alone, or IL-1β (0.3 to 3 ng/ml), TNFα (10 ng/ml) and IFN-γ (100 U/ml) in combination also failed to produce detectable nitrite/nitrate levels after 48 hours (n≥3). The rate of conversion of nitrate to nitrite using nitrate reductase was assessed by using known standards of sodium nitrate which were within the range of the Griess reaction (i.e. 1 - 20 μM), conversion rates were ~60% with a lower detection limit of 2 μM nitrate, when performed using Waymouths medium in the absence of FCS. Results were obtained from the conversion of supernatants from experiments performed in either Waymouths medium or DMEM in the absence of FCS. The presence of FCS proved problematic as it contained relatively high levels of nitrate (≥11 μM) which reduced the sensitivity of the conversions and caused large variations (>3 μM) across triplicates of the enzyme blank (i.e. nitrate reductase + NADPH + medium which has not been incubated with cells). Variation across triplicates of the enzyme blank within an experiment made result difficult to interpret. DMEM without phenol red also contains low levels of nitrate (1.6 μM) and subsequently the sensitivity of conversions performed in this medium were not as good as in Waymouths media, which contains no added nitrate.

Results obtained using the fluorimetric assay showed MC incubated with media alone, or stimulated with IL-1β (1, 3 ng/ml), TNFα (10, 300 ng/ml) or IFN-γ (100 U/ml) alone, or in combination showed no production of nitrite/nitrate above media control levels after 48 hours (n≥4). % conversion rates of nitrate to nitrite were generally 50 to 60%. However, the lower detection limit of the conversion was only 2 μM nitrate, despite the greater sensitivity of the fluorimetric assay (≥100 nM nitrite). The poor detection limit for nitrate was due to high backgrounds (1 to 2 μM) obtained with the enzyme blank, despite the use of Waymouths medium which contains no added nitrate. The high background may therefore be due to nitrate present in water and absorbed from glass and/or plasticware. Furthermore, variations of up to 1 μM in the levels of background nitrate measured in triplicate samples of the enzyme blank made results difficult to interpret.

6.2.3 Expression of mRNA for the inducible form of the NO synthase enzyme by cytokine activated human mesangial cells

Geller *et al* in 1993 were the first group to clone an inducible form of the NO synthase enzyme (iNOS) from human cells (hepatocytes) (Geller *et al.* 1993). Subsequent to this study, probes to detect human iNOS gene expression became available. Due to difficulties in demonstrating NO production by cytokine stimulated MC using nitrite/nitrate measurements, MC were probed for iNOS mRNA expression.

MC incubated in Waymouths medium alone, in the presence of 10% FCS did not express detectable iNOS mRNA transcripts. Surprisingly, stimulation of MC with a combination of IL-1β (1 ng/ml), TNFα (10 ng/ml) and IFN-γ (50 U/ml) for 8, 16 and 24 hours in the presence of 10% FCS resulted in time-dependent iNOS mRNA expression (data not shown).

The effect of cell cycle, FCS and different cytokine combinations on iNOS expression in MC was determined, in order to allow optimum conditions to be employed in future experiments.

(i) Cell cycle and FCS:

Figure 60 shows that non-quiescent MC stimulated with a cocktail of IL-1β (1 ng/ml), TNFα (10 ng/ml) and IFN-γ (100 U/ml) in the presence of 10% FCS (Fig. 60, C) express reduced levels of iNOS mRNA at 8, 24 and 48 hours compared to non-quiescent cells stimulated in the absence of FCS (Fig. 60, B), a 34 ± 2% (mean ± SEM, n=2) decrease in mRNA expression was observed after 24 hours stimulation in the presence of 10% FCS. Furthermore, cocktail stimulation of non-quiescent MC in the absence of FCS (Fig. 60, B) resulted in reduced iNOS mRNA expression of 35 ± 4% (mean ± SEM, n=2) at 24 hours compared to cells which were made quiescent before use (Fig. 60, A). All further experiments were therefore performed on quiescent MC, in the absence of FCS.

(ii) Cytokines:

The effect of different cytokines, either alone or in combination on iNOS mRNA expression was next assessed to determine the cytokine requirement for iNOS induction. iNOS mRNA transcripts were not detected in unstimulated MC. The proinflammatory

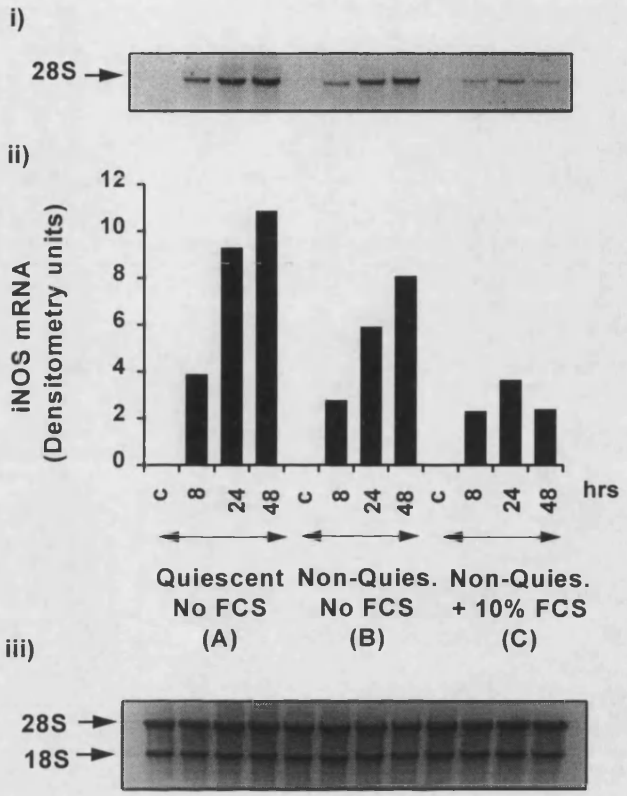


Figure 60. Effect of different culture conditions on iNOS mRNA expression in human MC stimulated with a cytokine cocktail. MC cultured in 25 cm^2 flasks were either preincubated in serum free medium for 24 hours (A), or left in 'complete' Waymouths medium prior to stimulation with a cytokine cocktail of IL-1β (1 ng/ml), TNFα (10 ng/ml) and IFN-γ (100 U/ml) for 48 hours, in either the absence (B) or presence (C) of 10% FCS. Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=2.

cytokines IL-1α, TNFα or IFN-γ added alone to MC did not induce detectable iNOS mRNA expression (n=3, data not shown). The combination of IL-1β/IFN-γ was the minimal requirement for iNOS mRNA expression, while other pairs of cytokines were ineffective (Fig. 61). The addition of TNFα did not significantly increase the IL-1β (1 ng/ml) / IFN-γ (100 U/ml) induced iNOS mRNA expression in MC (Fig. 61). Stimulation of MC with increasing doses of IL-1β (0.3 - 3 ng/ml) in the presence of 100 U/ml IFN-γ resulted in dose-dependent expression of iNOS mRNA (Fig. 62). No further increase in iNOS mRNA expression was observed with a higher dose of IL-1β (10 ng/ml). Similarly, stimulation with 30 to 300 U/ml of IFN-γ in the presence of IL-1β (1 ng/ml) resulted in dose-dependent iNOS mRNA expression at 48 hours in MC (Fig. 62). However, increasing concentrations of TNFα (3 to 30 ng/ml) in combination with IL-1β (1 ng/ml) and IFN-γ (100 U/ml) had no effect on the level of iNOS mRNA expression in MC (Fig. 62).

The time course of iNOS mRNA expression in MC following stimulation with IL-1β (1 ng/ml), TNFα (10 ng/ml) and IFN-γ (100 U/ml) was examined. In unstimulated cells iNOS transcripts were not detected at any of the time points examined. The cocktail of cytokines induced iNOS expression which was first detected at 4 hours, peaked at 24 hours and was still present at 72 hours (Fig. 63a). The levels of iNOS detected at 72 hours varied, depending on how viable the culture remained during the longer incubation periods with the cocktail, in the absence of FCS.

iNOS mRNA transcripts were consistently expressed in all the MC cultures tested (n=7) after stimulation with IL-1β and IFN-γ or IL-1β, IFN-γ and TNFα. However, culture supernatants from these same experiments contained undetectable levels of nitrite (<100 nM) and nitrate (<2 μM) measured by the fluorimetric assay, as represented in figure 63 (b).

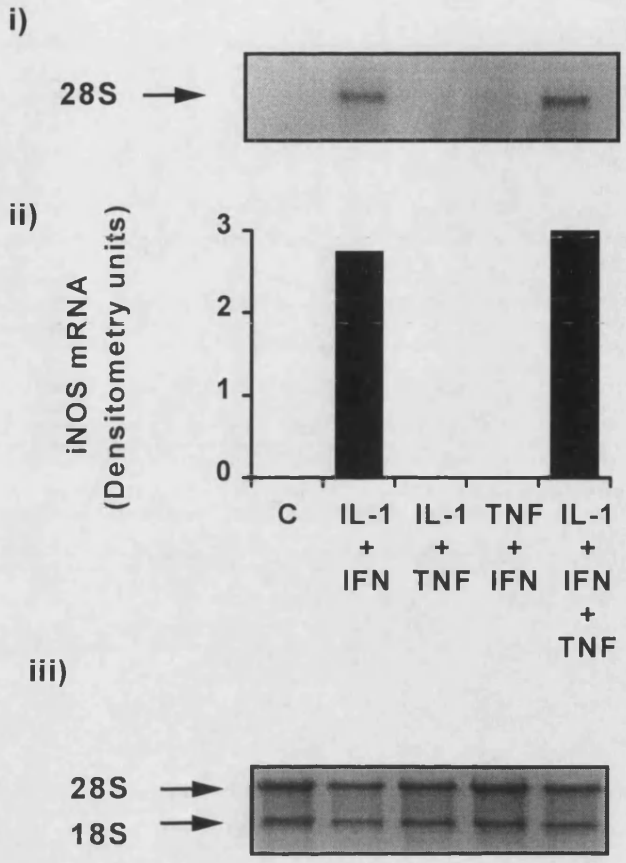


Figure 61. Specificity of iNOS mRNA induction in human MC. Cells cultured in 25 cm^2 flasks were stimulated with different combinations of the cytokines IL-1β (1 ng/ml), TNFα (10 ng/ml) and IFN-γ (100 U/ml), as indicated. After 48 hours, total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). The ethidium bromide stained gels showing the 18S and 28S ribosomal RNA were used to assess equal loading (iii). Representative blots are shown, n=2.

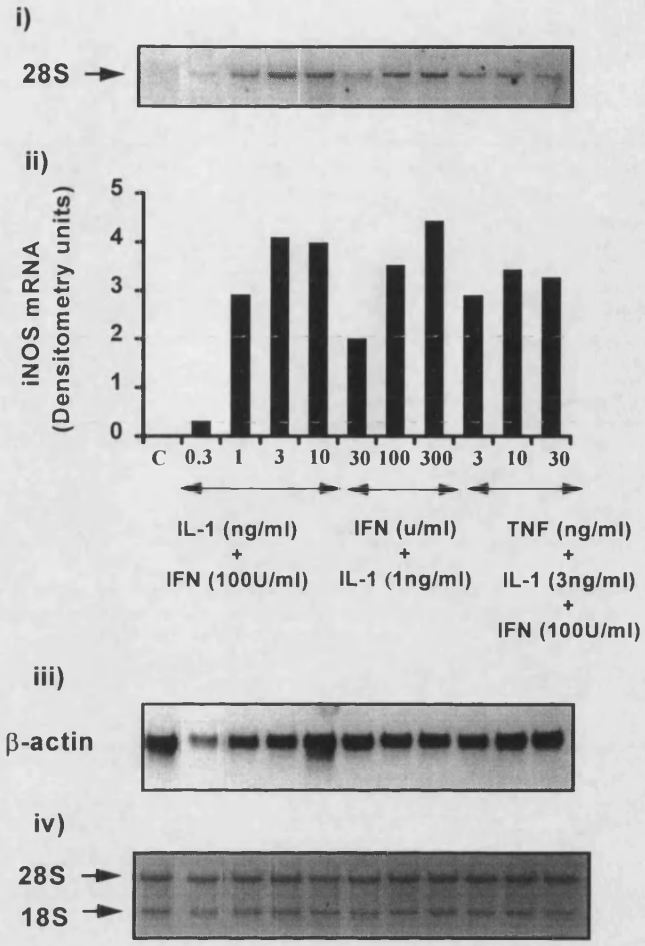


Figure 62. Dose-dependent expression of iNOS mRNA in cytokine stimulated MC. Cells were stimulated with either increasing doses of IL-1β (0.3 - 10 ng/ml) in the presence of 100 U/ml IFN-γ, IFN-γ (30 - 300 U/ml) in the presence of 1 ng/ml IL-1β or with TNFα (3 - 10 ng/ml) in combination with both IL-1β (1 ng/ml) and IFN-γ (100 U/ml). Total cellular RNA was extracted after 48 hours and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). Identical membranes were probed with the house-keeping gene β-actin (iii) to assess equal loading, together with the ethidium bromide stained gels showing the 18S and 28S ribosomal RNA bands (iv). Representative blots are shown, n=3.

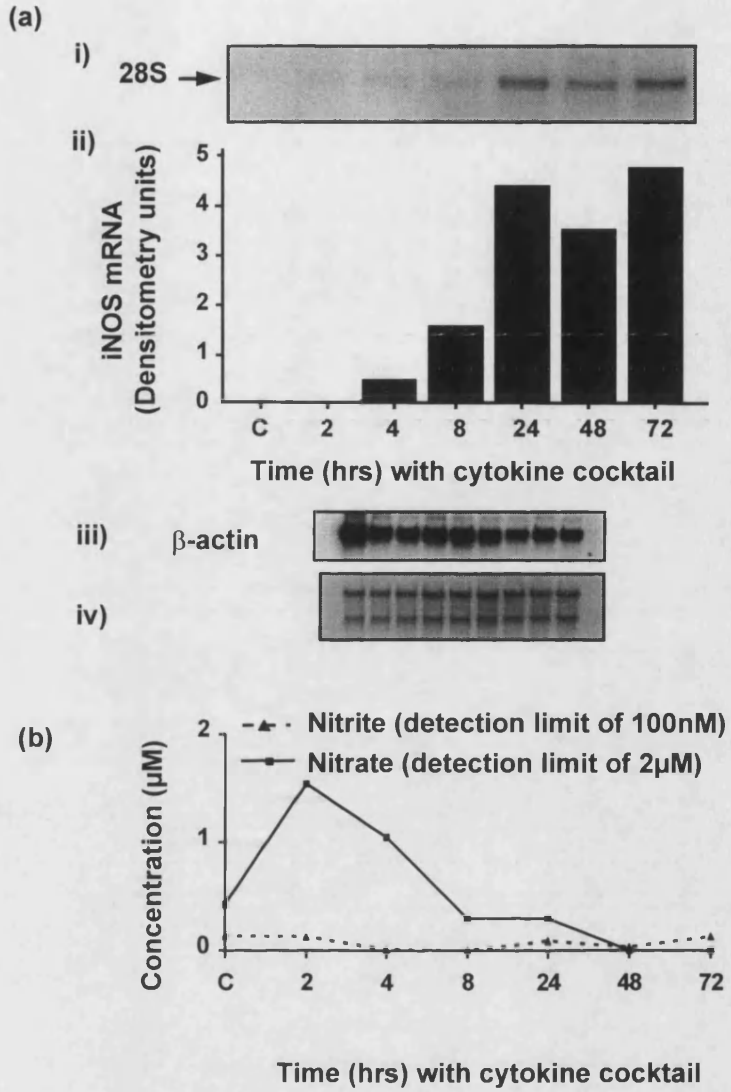


Figure 63. Time-dependent expression of iNOS mRNA in cytokine stimulated MC. Cells cultured in 25 cm^2 flasks were stimulated with 1 ng/ml IL-1β, 100 U/ml IFN-γ and 10 ng/ml TNFα for the times indicated.

(a) Total cellular RNA was extracted and northern analysis carried out. Autoradiographs (i) were analysed by densitometry and the results plotted using the absorbance units (ii). Identical membranes were probed with the house-keeping gene β-actin (iii) to assess equal loading, together with the ethidium bromide stained gels showing the 18S and 28S ribosomal RNA bands (iv).

(b) Nitrite and nitrate levels in 48 hour culture supernatants were measured using the fluorimetric assay.

A representative experiment is shown, similar results were obtained in 4 other experiments.

6.2.4. Inducible NOS protein expression by cytokine activated human mesangial cells

To determine whether the iNOS mRNA expressed by cytokine stimulated MC was being translated into protein, MC were probed for iNOS protein by western blot using a specific anti-iNOS antibody (monoclonal, human). Figure 64 (a) shows MC incubated with media alone for 48 hours contained undetectable amounts of iNOS protein. The combination of IL-1β (1 ng/ml), IFN-γ (100 U/ml) and TNFα (10 ng/ml) induced expression of a ~131 kDa protein after 24 and 48 hours. This protein corresponds to the molecular weight of the known form of human iNOS enzyme (Geller *et al.* 1993). Specificity of the iNOS antibody was confirmed by incubating an identical membrane with antisera containing 50 nM of a peptide corresponding to the C-terminal sequence of human iNOS, against which the antibody was directed. Addition of the blocking peptide completely inhibited staining (Fig. 64b).

iNOS protein was not detected in cells stimulated for 48 hours with either IL-1β (3 ng/ml), TNFα (300 ng/ml) or IFN-γ (100 U/ml) alone, or the combination of IL-1β (1 ng/ml) and TNFα (10 ng/ml) or TNFα (10 ng/ml) and IFN-γ (100 U/ml) (Fig. 65).

6.2.5. Effect of inhibiting superoxide generation on nitrite production by cytokine activated mesangial cells

The ability of cytokine activated human MC in culture to generate superoxide is well established (Radeke *et al.* 1990; Jones *et al.* 1993) and may result in nitrate as the end product of NO degradation, through formation of the peroxynitrite anion (Mulligan *et al.* 1991). However, the method used to assay nitrate levels in culture supernatants, using the enzyme nitrate reductase to convert nitrate to nitrite for quantitation using the Griess or fluorimetric nitrite assays had a poor lower detection limit (≥2 μM nitrate). The effect of blocking O_2^- production using an inhibitor of the NADPH-oxidase, 4-hydroxy-3-methoxyacetophenone (HMAP) (Suzuki *et al.* 1992) and the effects of the O_2^- scavengers, pyrrolidinedithiocarbamate (PDTC) and superoxide dismutase (SOD) on nitrite production by cytokine activated MC were therefore investigated.

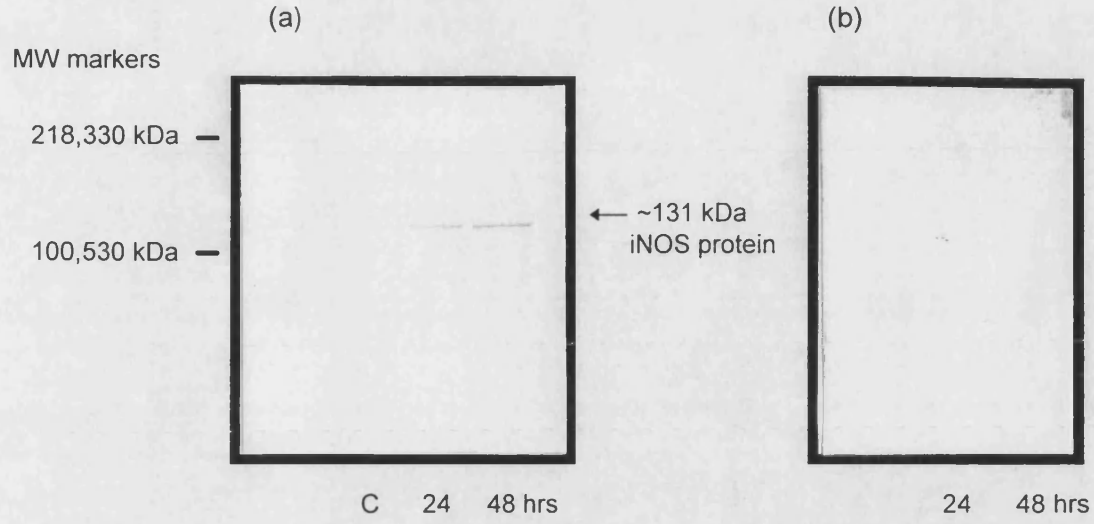


Figure 64. Production of iNOS protein by cytokine stimulated MC. Cells cultured in 25 cm^2 flasks were stimulated with 1 ng/ml IL-1β, 100 U/ml IFN-γ and 10 ng/ml TNFα for 24 or 48 hours. Control cells (C) were stimulated with media alone for 48 hours. Total cellular protein was extracted and resolved in a 7% SDS-polyacrylamide gel, prior to western blotting. iNOS protein was detected using a specific antibody directed against the human hepatocyte form of the inducible NOS enzyme, in either the absence (a) or presence (b) of a blocking peptide which corresponds to the C-terminal sequence of iNOS. Representative blots are shown, n=4.

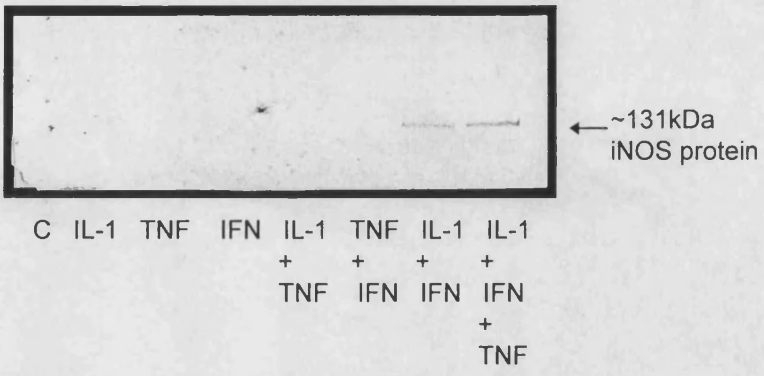


Figure 65. Specificity of iNOS protein production by cytokine stimulated MC. Cells were stimulated with IL-1β (1 ng/ml), TNFα (10 ng/ml) or IFN-γ (100 U/ml) either alone or in combination, as indicated. After 48 hours, total cellular protein was extracted and resolved in a 7% SDS-polyacrylamide gel, prior to western blotting and immunodetection. Representative blots are shown, n=2.

Pretreatment of MC with HMAP (3 to 300 μM) for 1 hour, or PDTC (3 to 1000 μM) or SOD (10 to 300 U/ml) for 30 minutes, prior to stimulation with IL-1β (1 ng/ml), IFN-γ (100 U/ml) and TNFα (10 ng/ml) resulted in undetectable nitrite levels (<100 nM) after 72 hours (data not shown, n=3).

6.2.6. Nitrite production by human MC: quantitation using chemiluminescence

The inability to detect increased nitrite production in culture supernatants from cytokine stimulated MC which positively express iNOS mRNA transcripts and protein may be due to lack of sensitivity of the fluorimetric assay. Chemiluminescence was therefore employed to quantitate nitrite levels in cell supernatants, the sensitivity of this assay is in the pM range. Figure 66 shows MC incubated with media alone generated 2.2 ± 0.2 nM (mean ± SEM, n=3) nitrite after 48 hours. Stimulation of MC with IL-1β (1 ng/ml), IFN-γ (100 U/ml) and TNFα (10 ng/ml) resulted in increased nitrite production of 4.2 ± 0.7 nM and 6.4 ± 0.2 nM (mean ± SEM, n=3) after 48 and 72 hours respectively.

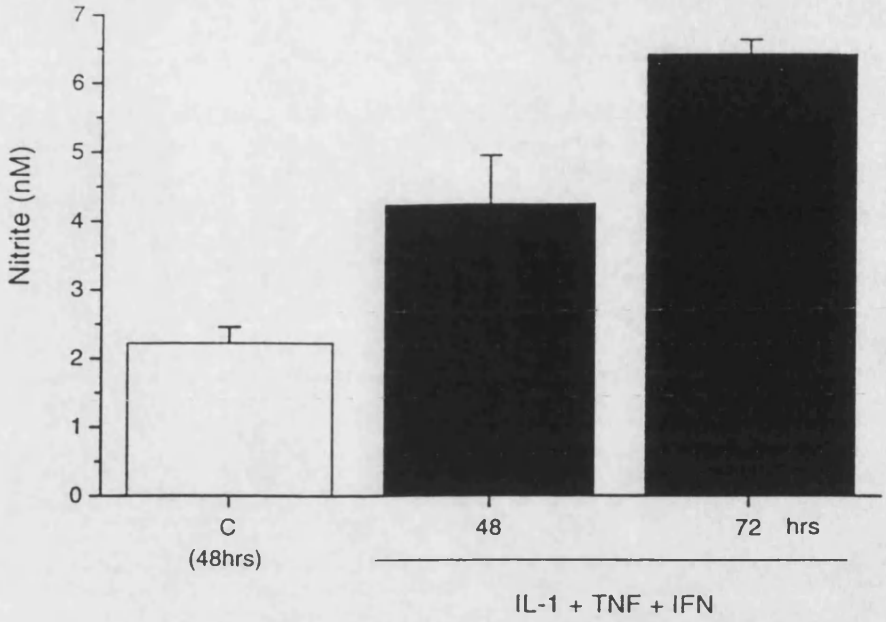


Figure 66. Chemiluminescence measurement of nitrite production by cytokine stimulated human MC. Cells cultured in 25 cm^2 flasks were stimulated with 1 ng/ml IL-1β, 100 U/ml IFN-γ and 10 ng/ml TNFα for 24, 48 or 72 hours. Control cells (C) were stimulated with media alone for 48 hours. Cell supernatants were quantitated for nitrite levels by chemiluminescence. Results are the mean ± SEM of n=3.

6.3. DISCUSSION

The presence of an inducible NO/L-arginine pathway in human cell types *in vitro* has, until recently been difficult to demonstrate. Nussler *et al* in 1992 demonstrated freshly isolated human hepatocytes produced an L-NMMA inhibitable increase in nitrite production and elevation of cGMP after stimulation with a 'cocktail' of factors, IL-1, TNF, IFN-γ and LPS (Nussler *et al.* 1992). The following year Geller *et al* (1993) cloned the human hepatocyte iNOS, which appeared to be a structurally distinct isoform, ~80% homologous to murine macrophage NOS and ~50% homologous to human endothelial NOS at the amino acid level. Unlike murine macrophage NOS, the activity of human hepatocyte NOS was partially inhibited by Ca^{2+} chelation or treatment with a calmodulin inhibitor (Geller *et al.* 1993).

In the present study the ability of cultured human mesangial cells to express the inducible NO/L-arginine pathway was investigated. MC expressed mRNA transcripts and protein for the human hepatocyte isoform of iNOS following stimulation with a combination of cytokines. The minimum cytokine requirement for detectable iNOS mRNA and protein expression was stimulation with a combination of IL-1 and IFN-γ. No other pair of cytokines, or individual cytokines tested proved to be effective for iNOS induction. These findings on cytokine specificity are consistent with reports in other human cell types which indicate that in contrast to animal tissues, induction of iNOS expression in human cells generally requires a combination of cytokines. The cytokine requirement for iNOS induction appears to vary across different cell types. Similar to our findings in MC, IL-1/IFN-γ was an effective stimulus for iNOS induction in human colonic epithelial cells (Kolios *et al.* 1995), human retinal pigmented epithelial cells (Goureau *et al.* 1994), human osteoblast-like cells (Ralston *et al.* 1994) and human lung epithelial cells (Asano *et al.* 1994). Modest induction of iNOS expression and/or activity was also observed in human osteoblast-like cells (Ralston *et al.* 1994) and human lung epithelial cells (Asano *et al.* 1994) after stimulation with TNF/IFN-γ, while human monocytes expressed iNOS mRNA following stimulation with IFN-γ/LPS (Reiling *et al.* 1994). The addition of TNF to the combination of IL-1 and IFN-γ was found to cause significant potentiation of nitrite production in these

studies (Kolios *et al.* 1995; Goureau *et al.* 1994; Ralston *et al.* 1994). However, stimulation of MC with increasing doses of TNF in the presence of sub-maximal doses of IL-1/IFN-γ caused no enhancement of iNOS mRNA levels, while the effect on nitrite production was not determined in these experiments. This finding supports the previous suggestion of Kolios *et al* (Kolios *et al.* 1995) that TNFα upregulates IL-1/IFNγ induced iNOS activity at a posttranscriptional level. In marked contrast to the human MC study and reports in other human cell types, induction of iNOS activity has been observed in human vascular smooth muscle cells (Junquero *et al.* 1992) and human chondrocytes (Charles *et al.* 1993; Maier *et al.* 1994) after stimulation with single cytokines, either IL-1, TNF or IFN-γ. Cloning of the human chondrocyte iNOS showed this isoform differed from the human hepatocyte iNOS by 7 amino acids, and unlike human hepatocyte iNOS, was insensitive to Ca^{2+} chelation and calmodulin inhibitors. It was therefore suggested that human hepatocyte iNOS and human chondrocyte iNOS may be distinct molecular forms, which are differentially regulated (Chartrain *et al.* 1994). In support of this, cloning of vascular smooth muscle, macrophage and hepatocyte iNOS isoforms from the same species (male Sprague Dawley rats) revealed some structural differences between the 3 isoforms which had previously been thought due to species variation (Mohaupt *et al.* 1994). This indicates different cell types may express structurally and functionally unique isoenzymes of iNOS, which may be encoded by a family of distinct iNOS genes.

Recent studies by Brown *et al* (1993b) and Andrew *et al* (1995) have indicated a role for NO in IL-1 and TNF induced IL-8 production, since NO donors and cGMP analogues upregulated, while L-arginine analogues partially inhibited IL-8 production in IL-1 stimulated human MC and TNF stimulated human melanoma cells. The role of NO in MC chemokine production was not investigated directly in the present study, but the findings of the cytokine requirement for expression of the human hepatocyte isoform of iNOS in MC indicate that this isoform is unlikely to play a role in IL-1 induced IL-8 generation, since single cytokines were not an effective stimulus for its induction. Recent studies have indicated an increasing number of human cell types (e.g. endothelial cells (Gross *et al.* 1991), monocytes/macrophages (Reiling *et al.* 1994) and lung epithelial cells (Asano *et al.* 1994)) can express both a constitutive and inducible

form of the NOS enzyme. Furthermore, a novel report in cultured rat MC demonstrated an ability of these cells to express both a designated vascular smooth muscle and macrophage iNOS isoenzymes, which were shown to be differentially regulated by TNF/IFN-γ stimulation (Mohaupt *et al.* 1994). An ability of human MC to express a number of NOS isoforms which are differentially regulated, may explain the reported effects of L-NMMA on IL-1 induced IL-8 production. In the present study, stimulation of MC with IL-1 alone did not induce a detectable increase in nitrite generation by MC using the fluorimetric nitrite assay, however any induced NO production may have been below the detection limit of the assay (<100 nM), and studies have shown 10 nM NO production is sufficient to induce elevation of intracellular cGMP and cause smooth muscle cell relaxation *in vitro* (Beckman & Tsai, 1994). Alternatively, the response may involve low output NO production by a constitutive NOS isoform. This area of work therefore requires further investigation.

Inducible NOS mRNA transcripts were first detected in MC 4 hours post-IL-1/IFN-γ/TNF stimulation, levels peaked at 24 hours and remained elevated beyond 72 hours. A similar time course of iNOS mRNA expression was observed in human colonic epithelial cells (Kolios *et al.* 1995), while human hepatocytes expressed peak mRNA levels 8 hours post-stimulation and levels had declined by 24 hours (Nussler *et al.* 1993). Stimulation of MC with cytokines in the presence of FCS was found to cause marked inhibition of iNOS mRNA expression. The inhibitory activity of FCS may be due, at least in part to platelet factors present in serum, since PDGF was previously shown to inhibit nitrite production in cytokine stimulated rat MC (Pfeilschifter, 1991a). Growth-arresting MC prior to cytokine stimulation in serum-free media may further increase iNOS mRNA expression by allowing sufficient time (24 hours) for the effects of serum factors to diminish prior to iNOS induction. Intriguingly, despite consistent detection of iNOS mRNA transcripts and iNOS protein in human MC isolated from different donors and at different passage numbers, the ability of MC to produce NO and thereby increase nitrite levels in cell supernatants was difficult to demonstrate, despite employing optimal experimental conditions by stimulating growth-arrested MC with a combination of IL-1/IFN-γ/TNF, in the absence of FCS. Nitrite production by cytokine-stimulated MC was not detected using the Griess reaction or a fluorimetric assay which

have lower detection limits of 0.8 μM and 100 nM nitrite respectively. Preliminary results obtained using a more sensitive chemiluminescence assay, which detects nitrite levels down to the pM range demonstrated stimulation of MC with IL-1/IFN-γ/TNF produced a two-fold increase in nitrite production after 48 hour of 4.2 nM nitrite. The synthesis of such small amounts of nitrite by MC explains the inability to measure nitrite production using the Griess or fluorimetric nitrite assay, due to their limited sensitivity. The small levels of nitrite produced by MC following cytokine stimulation does not exclude a role for NO in mesangial cell signalling, since 10 nM NO increased cGMP and causes smooth muscle cell relaxation *in vitro* (Beckman & Tsai, 1994). However, the production of such low levels of NO are uncharacteristic of an inducible isoform of NOS and contrast with studies in other human cell types, which describe the production of μM amounts of nitrite following cytokine stimulation, measured using the Griess reaction (Nussler *et al.* 1992; Goureau *et al.* 1994; Ralston *et al.* 1994; Asano *et al.* 1994). These findings suggest some factor(s) may be interfering with the conversion of NO to nitrite or restricting the activity of iNOS in MC in our system.

The low nitrite levels produced by MC are unlikely to be due to the conversion of synthesized NO to nitrate, as the use of nitrate reductase to convert nitrate to nitrite for measurement failed to detect nitrate production (≥2 μM) after 24, 48 or 72 hours stimulation with IL-1/IFN-γ/TNF. Furthermore the use of agents to block potential superoxide production by MC (Radeke *et al.* 1990) during the experiments, which can react with NO and lead to nitrate formation via the synthesis of the peroxynitrite anion (Mulligan *et al.* 1991), had no effect on the ability of mesangial cells to produce detectable nitrite levels (≥100 nM) after 72 hours.

The substrate for iNOS, L-arginine may also be metabolised to L-ornithine and urea by the action of arginase. Some cells can express both NOS and arginase activity indicating competition for substrate may occur (Cattell & Cook, 1993). Competition between arginase and NOS for L-arginine has been demonstrated *in vitro* in rat macrophages and in glomeruli isolated from rats with experimental GN, as addition of the NOS inhibitor, L-NMMA increased arginase activity, measured by the generation of radiolabelled urea from ^{14}C-labelled L-arginine (Cattell & Cook, 1993; Jansen *et al.*

1992). Studies have indicated that cultured mesangial cells are a major source of arginase (Cattell & Cook, 1993). However, limiting substrate did not account for the poor nitrite production by human MC in the present study, as increasing the arginine concentration of Waymouths medium from 360 μM to 2 mM for the duration of the experiment had no effect on nitrite production (<100 nM) by cytokine stimulated MC after 72 hours.

The findings of the present study are not in isolation. Morris and Billar (1994) have reported similar observations in human pulmonary artery smooth muscle cells and human cardiac myocytes which express significant iNOS mRNA and protein in response to a combination of IL-1/IFN-γ/TNF/LPS, while showing only minor increases in nitrite/nitrate production in cell supernatants. Human hepatocytes were found to generate significant nitrite/nitrate using the same experimental conditions. Furthermore, Reiling *et al* (1994) demonstrated iNOS mRNA expression in human monocytes stimulated with LPS/IFN-γ, but failed to detect nitrite production using the Griess reaction. This latter study highlights the difficulties researchers have experienced in demonstrating iNOS activity in human monocytes/macrophages (Schneemann *et al.* 1993; Sakai & Milstien, 1993) despite the proposed role of iNOS as an antimicrobial system in these cells, which has been firmly established in murine macrophages. Intriguingly, a recent study by Nicholson *et al* (1993) demonstrated human MC in culture produced μM amounts of nitrite after 24 hours stimulation with IL-1/IFN-γ or the more effective cytokine combination of IL-1/IFN-γ/TNF in the presence of 10% FCS, using the Griess reaction for nitrite measurement. The reason for the discrepancy between the present findings and those of Nicholson *et al* is unknown, since to my knowledge identical experimental conditions were tried during this study.

The poor nitrite production by human cells which express significant levels of iNOS mRNA and protein following cytokine stimulation may be due to a posttranslational defect in NO synthesis in these cells. iNOS activity may be affected at a posttranslational level by cofactor availability, since NOS enzymes require an array of biochemical cofactors to express activity (Morris & Billiar, 1994). Of particular interest is tetrahydrobiopterin (BH_4), a cofactor which may play a role in maintaining iNOS in

an active configuration. Baek *et al* (1993) have shown BH_4 to be important in forming active dimeric enzymes from inactive iNOS monomers. BH_4 is synthesized by cells *de novo* from GTP or produced by the activities of a salvage (recycling) pathway. The *de novo* synthesis of BH_4 was found to be co-induced with iNOS in a host of cells, including rat aortic smooth muscle cells (Gross & Levi, 1992) and rat mesangial cells (Muhl & Pfeilschifter, 1994) following cytokine stimulation, and *de novo* BH_4 synthesis was found to be an absolute requirement for NO generation. Furthermore, addition of sepiapterin to increase intracellular BH_4 levels via the salvage pathway dose-dependently augmented LPS or cytokine induced NO synthesis in these cells, indicating BH_4 levels under normal culture conditions was limiting the rate of NO production (Gross & Levi, 1992; Muhl & Pfeilschifter, 1994). An inability of certain cells in culture to synthesize sufficient BH_4, which may be acquired from other cell types *in vivo*, or be an artefact of cell culture, could therefore account for their inability to produce sufficient NO and thus nitrite *in vitro*. The effect on nitrite production of increasing BH_4 levels in cultured human MC remains to be determined.

Some factor(s) produced by cytokine stimulated human MC may inhibit iNOS activity at a posttranslational level and thus effect nitrite production in this system. A number of cytokines have been shown to exert inhibitory effects on nitrite synthesis, including TGFβ, IL-8 and IL-10 (Ding *et al.* 1990; Cunha *et al.* 1992; McCall *et al.* 1992). These factors however appear to inhibit an early event in the induction of iNOS activity, since the cytokines were only inhibitory when added prior to or simultaneously with the stimuli for iNOS expression, and were without effect if added 6 hours later. The ability of human MC to synthesize some of these cytokines may not therefore account for the low levels of nitrite production. In addition to the production of inhibitory cytokines, methylated arginine analogues which inhibit iNOS activity have been found in the plasma and urine of patients with end-stage chronic renal failure demonstrating endogenous production of these inhibitors (Vallance *et al.* 1992). Cellular sources however, have yet to be identified.

The findings of the present study indicate complex regulation of iNOS activity in human MC. iNOS activity appears to be regulated both at the level of iNOS mRNA induction

(e.g. by FCS) and at a posttranslational level, since expression of significant iNOS mRNA and protein by MC was not sufficient for the synthesis of high levels of nitrite which is a characteristic of this isoform of NOS. Further studies into the regulation of human MC iNOS activity are therefore required, since experimental conditions in terms of the appropriate repertoire of cytokines, growth factors, cofactors, etc., which are necessary to support high output NO synthesis *in vitro* have not yet been fully defined.

7. CONCLUSIONS AND IMPLICATIONS

Human MC are now recognised as a potentially important source of inflammatory mediators, including members of the recently identified chemokine superfamily. A novel finding of this study was the ability of cultured human MC to express and secrete the specific chemotactic factor for CD45RO/$CD4^+$ T lymphocytes, RANTES, following stimulation with the proinflammatory cytokines IL-1 and TNF. MC-derived RANTES may play a potential role in the recruitment of specific T cell subsets during glomerular injury, since T lymphocytes have been detected in glomeruli at both an early and late stage of the disease process in animal models of immune complex mediated GN (Tipping *et al.* 1985; Radounikli *et al.* 1995). The infiltrating T lymphocytes were predominantly $CD4^+$ positive. T lymphocytes of this subset may play an important role in both the initiation and perpetuation of the immune response (Holdsworth & Tipping, 1991).

A recurrent finding throughout this work was that expression of the chemokines IL-8, MCP-1 and RANTES by cytokine stimulated human MC was differentially regulated at several levels. Expression of the chemokines differed in terms of (a) kinetics of expression, (b) specificity of induction, (c) susceptibility to regulation by the intracellular second messenger, cAMP and (d) the involvement of secondary proteins in TNF induced chemokine gene expression. This ability of the chemokines to be differentially regulated may be important for the fine tuning of an immune response, and expression of each chemokine *in vivo* will be dependent on the net regulatory effects of local inflammatory mediators (e.g. IL-1, TNF, PGE_2, IFN-γ). Furthermore, these findings provide support for the suggestion that redundancy in the chemokine superfamily may be more apparent than real, since expression of members possessing overlapping chemotactic activities may be regulated temporally, as well as spatially and vary in potency for the given target cell (Schall, 1994).

The ability of IL-1 and TNF to induce expression and production of the same chemokines in MC, with different kinetics was a surprising finding. IL-1 induced IL-8,

MCP-1 and RANTES gene expression was more tightly regulated than the TNF response, since mRNA levels declined rapidly after maximal expression of the mRNA transcripts. TNF induced chemokine expression, in comparison remained sustained. Interestingly similar differences in the kinetics of IL-1 and TNF induced chemokine expression have since been observed in human vascular smooth muscle cells (Dr N. Jordon, personnel communication). The significance of this finding is not yet clear, however tighter regulation of the IL-1 response in tissue cells, such as MC may be due to the fact that IL-1 can be expressed by a wide range of immune and non-immune cells following appropriate stimulation, while expression of TNF is limited mainly to macrophages and T lymphocytes (Akira *et al.* 1990a). TNF may consequently only be present during a 'developed' inflammatory response.

The ability of agents that elevate intracellular cAMP to differentially regulate IL-6, IL-8 and RANTES production in IL-1 or TNF stimulated human MC argues against a role for cAMP as a major second messenger in mediating IL-1 or TNF signal transduction. However these findings, together with other published studies indicate cAMP is an important regulator of IL-1 and TNF activity, and cytokine expression in general. The effects of cAMP elevation on MC cytokine production is summarized in table 8, which includes the work presented in this thesis, together with the findings of Satriano *et al* (1993) and Rovin and Tan (1994) relating to MCP-1 production. Similar studies have demonstrated differential regulation of T lymphocyte-derived cytokines by cAMP-elevating agents, production of IL-2 was downregulated, IL-3 and IL-4 were unchanged, while IL-5 was increased (Snijdewint *et al.* 1993). Interestingly, a role for the guanine nucleotide, cGMP in regulating IL-1 induced IL-8 production in human MC (Brown *et al.* 1993b) and IL-3 production by T lymphocytes has also been suggested (Marcinkiewicz & Chain, 1993), although cGMP had opposing effects to cAMP. Thus a balance between the intracellular levels of these two nucleotides may be involved in a fine tuning of cytokine release. *In vivo*, the intracellular levels of cAMP and cGMP in human MC, and other cell types may be regulated by endogenous or exogenous PGE_2 and NO production.

Effect of cAMP on IL-1 induced cytokine production		Effect of cAMP on TNF induced cytokine production	
IL-6	↑	IL-6	↑
IL-8	→	IL-8	→
RANTES	→	RANTES	↓
MCP-1	↓	MCP-1	↓

Table 8. Summary of the effects of elevation of intracellular cAMP on IL-1 or TNF induced cytokine production in MC.

The present study demonstrates an ability of human MC to express the inducible nitric oxide synthase enzyme. In comparison to rodent MC, iNOS mRNA and protein expression was only induced in human MC following stimulation with a combination of cytokines, IL-1 and IFN-γ, or IL-1, IFN-γ and TNF. The regulation of iNOS activity in human MC however, appears complex, since despite significant iNOS mRNA and protein expression, production of NO could not be readily detected using a range of nitrite assays. Intriguingly, my findings contrast with two recent reports which describe μM amounts of nitrite production by human MC following stimulation with a combination of IL-1, IFN-γ and TNF (Nicolson *et al.* 1993; Saura *et al.* 1996). Clearly, further investigations into the regulation of iNOS activity in human MC is required, and until the isoforms of NOS expressed by MC and their regulation has been characterised, the potential role of NO as an endogenous regulator of chemokine expression in human MC remains unresolved.

The bacterial agent, pertussis toxin was found to inhibit both IL-1 induced IL-8 and IL-6 production in MC. This property of the toxin appeared to be unrelated to its ability to ADP-ribosylate and inactivate $G_{i/o}$ type proteins. Furthermore, cholera toxin, the bacterial agent which activates the cAMP signal transduction pathway through modification of G_s by ADP-ribosylation was found to upregulate MC cytokine production by both cAMP dependent and independent mechanisms. Taken together, these findings suggest that while the toxins remain useful tools for investigating signal transduction pathways, their mechanism of action is not specific and thus care must be taken when interpreting their effects in a system. Use of the control reagent, the B oligomer is paramount in studies which employ these toxins to provide some indication whether their mechanism of action is G protein dependent.

Renewed interest in the use of phosphodiesterase inhibitors as a therapeutic approach to certain inflammatory disorders has stemmed from the observations that PDE inhibitors can effectively suppress TNF production in endotoxin stimulated human mononuclear cells (Semmler *et al.* 1993b; Semmler *et al.* 1993a; Schade & Schudt, 1993) and IL-6 production in IL-1 stimulated human lung fibroblasts (Zitnik *et al.* 1993), via elevation of intracellular cAMP. The findings presented in this thesis support a pharmacological

potential for PDE inhibitors, since agents which elevated intracellular cAMP selectively modified human MC cytokine production. These agents exerted antiinflammatory effects by suppressing expression of the C-C chemokines, RANTES and MCP-1 (Rovin & Tan, 1994; Satriano *et al.* 1993) in cultured MC, thus these agents may act to reduce monocyte and T lymphocyte glomerular infiltration *in vivo*. In addition, the cAMP-elevating agents significantly upregulated MC IL-6 production, a cytokine with both proinflammatory and antiinflammatory properties. The regulatory effects of cAMP on cytokine production however, appear to be cell specific, since cAMP-elevating agents had opposing effects on IL-1 induced IL-6 production in human MC and human lung fibroblasts (Zitnik *et al.* 1993). Thus the beneficial effects of such treatment will be dependent upon the target cells, the importance of individual cytokines in the disease process and the net effect of cAMP elevation on the cytokine network.

The findings presented in this thesis lend support to the hypothesis that activated MC play a direct role in the initiation and propagation of inflammatory events within the glomerulus via the generation of inflammatory mediators. The release of chemokines by intrinsic glomerular cells may be important in initiating an influx of leukocytes into the injured tissue, as well as contributing to the progression of inflammatory renal injury. Since chemokines appear to be relatively selective in their actions compared with many other inflammatory mediators, they have been regarded as promising targets for the development of anti-inflammatory therapies. Understanding the intracellular mechanisms that regulate chemokine production in glomerular cells will enhance the design of effective therapeutic strategies for the treatment of glomerular inflammation.

8. PUBLISHED WORK

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ABSTRACTS

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APPENDIX I. GENERAL BUFFERS

Unless otherwise stated all standard laboratory reagents were obtained from Fisons plc (Loughborough, UK), BDH Laboratory supplies (Poole, UK) or Sigma Chemical Co. (Poole, UK).

Phosphate Buffered Saline (PBS), pH 7.4

140 mM NaCl

2.7 mM KCl

1.5 mM KH_2HPO_4

8.1 mM Na_2HPO_4

1 M Potassium phosphate buffer, pH 7.5

0.7 M K_2HPO_4

0.3 M KH_2PO_4

0.15 M Potassium phosphate buffer, pH 7.5

0.105 M K_2HPO_4

0.045 M KH_2PO_4

100 mM HEPES buffer, pH 8.5

1 M HEPES buffer solution (Gibco BRL, Paisley, UK) was diluted 1:10 in double-distilled water.

APPENDIX II. CELL CULTURE REAGENTS

All solutions for cell culture were prepared using Steripak sterile, pyrogen-free distilled water (Steripak Ltd, Cheshire, UK).

1x PBS, pH 7.4

10x sterile PBS (without Ca^{2+} and Mg^{2+}) (Gibco) was diluted 1:10 in water. No need to pH.

Hanks Balanced Salts Solution (HBSS), pH 7.4 (for MC isolation, see 2.4.1)

100 ml 10x Hanks' balanced salts (Gibco)
6 ml 7.5% sodium bicarbonate (Gibco)
20 ml 1 M HEPES buffer solution (Gibco)
200 µl 40% (w/v) NaOH
500 µl 10 mg/ml Gentamicin (Gibco)
Make up to 1 L with distilled water. No need to pH.

Culture media

Waymouths medium MB752/1 (Gibco) was supplemented with penicillin-streptomycin (10 U/ml and 10 µg/ml, respectively) (Gibco) and fungizone (0.5 µg/ml) (Gibco) before use.

Dulbecco's Modified Eagles Medium (DMEM), without phenol red (used during nitrite determinations) was supplemented with penicillin-streptomycin (10 U/ml and 10 µg/ml, respectively), fungizone (0.5 µg/ml) and 2 mM glutamine (Gibco) before use.

Foetal Calf Serum (FCS)

FCS (Gibco) was heat-inactivated at 56°C for 30 minutes and stored in aliquots at -20°C.

1% Gelatin solution

2% gelatin solution, type B from bovine skin (cell culture grade) (Sigma) was diluted 1:1 with water.

APPENDIX III. ELISA BUFFERS

PBS coating buffer, pH 7.4 (For IL-6 ELISA)

140 mM NaCl

2.7 mM KCl

1.5 mM KH_2HPO_4

8.1 mM Na_2HPO_4

Carbonate coating buffer, pH 9.6 (For IL-8 and RANTES ELISA)

15 mM Na_2CO_3

35 mM $NaHCO_3$

IL-6 wash buffer, pH 7.2

2.5 mM NaH_2PO_4

7.5 mM Na_2HPO_4

0.5 M NaCl

0.1% (v/v) Tween-20

IL-8 / RANTES wash buffer, pH 7.5

140 mM NaCl

2.7 mM KCl

1.5 mM KH_2HPO_4

8.1 mM Na_2HPO_4

0.05% (v/v) Tween-20

0.1 M Citric acid-phosphate substrate buffer, pH 5 (For IL-6 and RANTES ELISA)

34 mM citric acid

66 mM Na_2HPO_4

Diethanolamine substrate buffer, pH 9.8 (For IL-8 ELISA)

10% (v/v) diethanolamine (BDH)

1 mM $MgCl_2$

Store in the dark, at 4°C.

APPENDIX IV. REAGENTS FOR NORTHERN ANALYSIS

DEPC-treated water or buffer

1 ml of diethyl pyrocarbonate (DEPC) (Sigma) was added per litre of double-distilled water or buffer and incubated overnight at 37°C. Autoclave (15-20 lb/in ^{2} for 20 minutes at 121°C).

0.75 M Sodium citrate solution, pH 7.0

0.75 M trisodium citrate dihydrate
in double-distilled water. Autoclave.

20% (w/v) SDS solution

20 g SDS dissolved in 100 ml sterile double-distilled water.

2 M TRIS-HCl stock solution, pH 8

2 M Tris(hydroxymethyl)aminomethane (TRIS)
in double-distilled water. Autoclave.

0.5 M EDTA stock solution, pH 8

0.5 M Ethylenediamine-tetraacetic acid, disodium salt (EDTA)
in double-distilled water. Autoclave.

Nucleic acid extraction buffer

4 M guanidine thiocynate (Fluka, Gillingham, UK)
25 mM sodium citrate (17.6 ml of 0.75 M stock/500 ml)
0.5% sarcosyl (8.3ml of 30% stock (BDH)/500ml)
in sterile double-distilled water.

Add 0.1 M 2-mercaptoethanol (0.36 ml/50 ml) prior to use. Use buffer containing 2-mercaptoethanol within 1 month. Store at 4°C.

Phenol extraction buffer

100 mM TRIS	(25 ml of 2 M stock/500 ml)
10 mM EDTA	(10 ml of 0.5 M stock/500 ml)
1% SDS	(25 ml of 20% stock/500 ml)

in double-distilled water. Autoclave.

3M Sodium acetate, pH 5.2

3 M sodium acetate dissolved in 250 ml double-distilled water.

Adjust pH to 5.2 using 3 M glacial acetic acid (requires approximately 200 mls)

DEPC-treat and autoclave.

75% (v/v) Ethanol solution

75 ml absolute ethanol (Hayman Ltd, Witham, UK) in 25 ml DEPC-treated water

20X MOPS running buffer

0.4 M 3-[N-morpholino]propane-sulphonic acid (MOPS)

0.02 M EDTA	(4 ml of 0.5 M stock/100 ml)
0.2 M sodium acetate	(6.64 ml of 3 M stock/100 ml)

Adjust volume to 100 ml with double-distilled water and pH with ~ 0.85 g solid NaOH.

Filter sterilize.

1x MOPS running buffer

50 ml 20x MOPS in 950 ml DEPC-treated water.

1 mg/ml Ethidium bromide solution

10 mg ethidium bromide (Sigma) in 10 ml DEPC-treated water

RNA Sample buffer (per sample)

7 µl formaldehyde

4 µl 20x MOPS running buffer

2 µl 1 mg/ml ethidium bromide solution

20 µl formamide (mol. biol. grade, BDH)

Made up fresh immediately before use.

Bromophenol blue solution

0.001 g bromophenol blue

3 ml glycerol

Adjust volume to 10 ml with DEPC-treated water.

20X SSC, pH 7

3 M NaCl

0.3 M trisodium citrate dihydrate

in double-distilled water. DEPC-treat and autoclave.

Buffer 1

0.1 M maleic acid

0.15 M NaCl

Adjust to pH 7.5 with ~ 75 g solid NaOH. DEPC-treat and autoclave.

Blocking stock solution

10 g (w/v) blocking reagent (Boehringer Mannheim, Lewes, UK) in 100 ml of buffer 1.

Microwave for 2 short bursts to dissolve. Autoclave.

Hybridization solution

5X SSC	(25 ml of 20X stock/100 ml)
0.1% sarcosyl	(0.33 ml of 30% stock/100 ml)
0.02% SDS	(0.1 ml of 20% stock/100 ml)
1% blocking buffer	(10 ml of 10% stock/ 100 ml)

in DEPC-treated water.

2X SSC , 0.1% SDS solution

50 ml of 20X SSC

2.5 ml of 20% SDS stock

Adjust volume to 500 ml with DEPC-treated water.

0.1X SSC, 0.1% SDS solution

2.5 ml of 20X SSC

2.5 ml of 20% SDS stock

Adjust volume to 500 ml with DEPC-treated water.

Washing buffer

0.3% (v/v) Tween-20 in buffer 1

Buffer 2

10% (v/v) blocking stock solution in buffer 1

Buffer 3, pH 9.5

0.1 M TRIS

0.1 M NaCl

in sterile double-distilled water.

APPENDIX V. REAGENTS FOR SDS-PAGE AND WESTERN BLOTTING

1.5 M TRIS-HCl, pH 8.8

1.5 M TRIS

in double-distilled water.

0.5 M TRIS-HCl, pH 6.8

0.5 M TRIS

in double-distilled water.

10% (w/v) SDS solution

5 g of SDS in 50 ml double-distilled water.

10% (w/v) Ammonium persulphate solution

0.1 g ammonium persulphate in 1 ml double-distilled water.

Made up fresh on day

Electrophoresis buffer

25 mM TRIS

192 mM glycine

0.1% SDS

1X Electrophoresis sample buffer

1 ml 1.5 M TRIS-HCl, pH 8.8

0.8 ml glycerol

1.6 ml 10% SDS solution

0.4 ml 2-mercaptoethanol

4.2 ml double-distilled water

0.01 g bromophenol blue (Bio-Rad)

Sample buffer was stored at room temperature for up to 1 week.

Coomassie blue stain

40% (v/v) methanol

7% (v/v) acetic acid

0.1% (w/v) coomassie brilliant blue R-250 (Sigma)

in distilled water

Destain solution

40% methanol

7% acetic acid

in distilled water.

Transfer Buffer

25 mM TRIS

192 mM glycine

20% methanol

APPENDIX VI. DRUG SOLUTIONS

CYTOKINES

Recombinant human IL-1α (specific activity $3x10^8$ U/mg), a gift from Hoffman La Roche (Nutley, New Jersey) was diluted in sterile PBS + 0.25% (w/v) bovine serum albumin (BSA, low endotoxin) to 10 μg/ml and stored in aliquots at -20°C.

Recombinant human IL-1ß (specific activity $1.2x10^7$ U/mg), a gift from Glaxo (Greenford, UK) was diluted in PBS + 0.25% BSA to 1 μg/ml and stored in aliquots at -20°C.

Recombinant human TNFα (specific activity $6x10^7$ U/mg), a gift from Bayer (Slough, UK) was diluted in PBS + 0.1% BSA to 100 μg/ml and 500 μg/ml and stored in aliquots at -70°C.

Recombinant human IFN-γ (specific activity $>2.0x10^7$ U/mg) was purchased from Boehringer Mannheim and stored in aliquots at -20°C.

Recombinant human IL-1 receptor antagonist (IL-1ra) was supplied by Synergen INC (Boulder, Colorado, USA) and stored at 5 mg/ml in PBS + 0.25% BSA in aliquots at -20°C.

All cytokines were further diluted in Waymouths medium immediately prior to use.

COMPOUNDS WHICH ELEVATE INTRACELLULAR cAMP

Forskolin (Sigma) was dissolved in dimethylsulphoxide (DMSO, cell culture grade, Sigma) to 100 mM and stored in the dark at room temperature. This stock was further diluted in Waymouths medium immediately prior to use. To prevent precipitation, it was necessary to initially add the forskolin to a few ml of Waymouths medium with agitation, before further diluting.

Dibutyryl cAMP (Db-cAMP) (Sigma) was dissolved in sterile distilled water to a concentration of 5 mg/ml and stored in aliquots at -20°C. Further dilutions were made in Waymouths medium immediately prior to use.

3-Isobutyl-1-methyl-xanthine (IBMX) (Sigma) was dissolved in warmed (37°C) Waymouths medium to a concentration of 1 mM on the day required. Further dilutions were made in Waymouths medium.

RO 20-1724 (Calbiochem, Nottingham, UK) was dissolved in DMSO to a concentration of 200 mg/ml and stored in aliquots at -20°C. Further dilutions were made in Waymouths medium immediately prior to use.

BACTERIAL TOXINS

Cholera toxin (CT) (Gibco), **cholera toxin B oligomer** (CT-B) (Gibco and Sigma), **pertussis toxin** (PT) (Gibco) and **pertussis toxin B oligomer** (PT-B) (Gibco) were reconstituted in sterile distilled water to 1 mg/ml., 0.5 mg/ml, 0.1 mg/ml and 1 mg/ml respectively and stored at 4°C for either 2 months (PT, PT-B) or 6 months (CT, CT-B).

Recombinant pertussis toxin (55 μg/ml stock in PBS) was a gift from Dr Luke O'Neill, Trinity College, Dublin and was made by Sclavo Ltd (Sienna, Italy).

Mutated pertussis toxins, PT/R_9E and PT/R_9ENK (300 and 150 μg/ml in PBS respectively) were a gift from Dr Yves Lobet, SmithKline Beecham, Rixensart, Belgium. All toxins were further diluted in Waymouths medium immediately prior to use.

MISCELLANEOUS COMPOUNDS

Lipopolysaccharide (LPS) (serotype 0111:B4) (Sigma) was reconstituted in sterile PBS and stored in aliquots at -20°C

Indomethacin (Sigma) was dissolved in absolute ethanol to a concentration of 10 mg/ml on the day required. Further dilutions were made in Waymouths medium .

Actinomycin D (AcD) (Sigma) was dissolved in DMSO to 5 mg/ml and stored in aliquots at -20°C for a maximum of 1 month. Further dilutions were made in Waymouths medium immediately prior to use. Aliquots were then discarded.

Cycloheximide (CHX) (Sigma) was dissolved in Waymouths medium immediately prior to use.

Phalloidin-FITC conjugated (Sigma) was dissolved in PBS to 0.5 mg/ml and stored in aliquots at -20°C. Further dilutions were made in PBS immediately prior to use.

4-hydroxy-3-methoxyacetophenone (HMAP or acetovanillone) (Aldrich chemical Co. Ltd, Gillingham, UK) was dissolved in warmed (37°C) Waymouths medium to 1.2 mM on the day required. Further dilutions were made in Waymouths medium.

Superoxide dismutase (SOD) (Sigma) was dissolved in PBS to 15,000 U/ml and stored in aliquots at -20°C. Further dilutions were made in Waymouths medium immediately prior to use.

Pyrrolidinedithiocarbamate (PDTC) (Sigma) was dissolved in Waymouths medium to 0.1 M on the day required. Further dilutions were made in Waymouths medium.